SEPTICAEMIA, THE ADRENAL GLAND, AND LOW GRADE DIPHTHERIA ANTITOXIN

by

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The adrenal gland was originally held to possess a detoxicating function in infections, but this conception fell into disfavour for a number of years. Now, however, opinion favours a modified view of the original hypothesis and interest in the adrenal gland in infections has been re-awakened.

One of the earliest instances of the relationship of infection to the adrenals was in 1895, when Roux in France and Behring in Germany found that the first organ affected by diphtheria toxin in the guinea pig was the adrenal gland.

It is generally recognized now that most infections have a direct effect on the adrenal. It is still a debatable point, however, whether the adrenal reaction is due to hypersensitivity to toxins or whether it is due to attempt on the part of the gland to combat the toxins circulating in the body.

THE RELATIONSHIP OF THE FATS AND LIPOIDS OF THE ADRENAL CORTEX TO INFECTIONS.

A number of workers have investigated this subject, among them being Weltmann (1913), Elliott (1906, 1912, and 1914), Goldzieher (1911), Stewart and Rogoff (1916, etc.), and Whitehead (1932), Deanesly (1931), and Hoerr (1931) in more recent years.

Weltmann investigated the changes of the lipoid substances of the adrenal in some eighty or more diseases. In puerperal sepsis, ordinary sepsis, and post abortal sepsis, he found that the adrenal rapidly lost its lipoids from all three zones; Elliott also found that in various infective conditions there was a reduction in the stainable fatty substances of the cortex. In 1906, Elliott and Tuckett showed that as the lipoids of the adrenal cortex decreased the amount of fat stainable with osmium tetroxide increased.

Baumann and Holly (1923) found that in acute infections the cholesterol content of the adrenal decreased and that there was a slight increase of fat.

Clevers and Goormaghtigh (1922) similarly found a reduction or disappearance of cholesterol and lipoids from the adrenal after a lethal dose of diphtheria toxin, while the fatty substances of the adrenal increased.

Cramer (1916, etc.) has shown that in streptococcal infections of varying virulence there is partial depletion of adrenalin but no effect on the lipoids when the animals receive a sub-lethal infection. In cases where the animals died, the adrenal cortex was found to be completely depleted of lipoids.

Deanesly (1931) did not consider that the depletion of the lipoids in animals was the actual cause of death, for she found that some animals which died possessed considerable amounts of lipoidal material in the cortex, whereas others which survived frequently possessed less. Also she found that the preliminary treatment with thyroxin which tended to cause an efflux of lipoids from the adrenal cortex did not increase the susceptibility of the animals to infection.

This work was conducted in an endeavour to elucidate some of the problems associated with the treatment of acute infective conditions, particularly the treatment of puerperal septicaemia with low-grade diphtheria antitoxin per os.

Campbell (1926) mentions that this treatment was introduced by Paton some years ago, and in this paper he points out that with the present state of knowledge the defence of serum therapy was a defence of empirical medicine. It is with the object of reducing this empirical use of the antitoxin to a more scientific basis that Dr. Campbell and the present author initiated a series of experiments at the Baker Research Institute, Melbourne.[1]

It is not proposed to go into details of the clinical treatment of sepsis with the antitoxin. Campbell mentions some outstanding cases, and he has personally communicated a very large number since his paper was written.

EXPERIMENTAL RESULTS.

For this work, rabbits and mice were used, and the most significant results were obtained from the former.

Experiments with Rabbits.

Eight rabbits were each injected intravenously with 4 c.c. of a suspension of virulent streptococci in normal saline.

Three of these animals were given no treatment of any kind. Two were given a daily subcutaneous injection of low grade diphtheria antitoxin. Three were given quantities of serum by mouth.

The first day following the injection of the streptococci, an animal receiving serum by injection, and one receiving no treatment, were killed and the adrenals removed. On the second day an untreated animal and an animal receiving serum by mouth were similarly treated. On the third day two more animals, one receiving serum by mouth and the other serum subcutaneously,

[1] In these experiments we were given invaluable assistance by Miss Hildred Butler, Bacteriologist to the Baker Institute. The final experiments for this work were carried out by the present author in the Department of Experimental Biology and Physiology, Australian Institute of Anatomy.

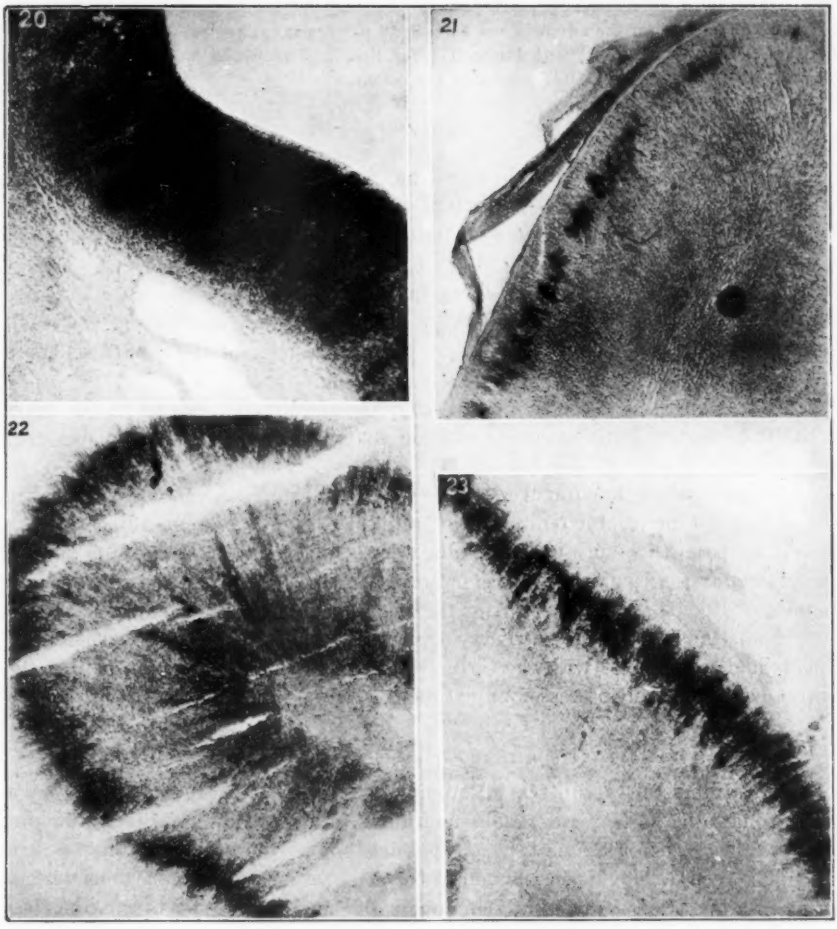

Fig. 20. Adrenal gland of rabbit with septicaemia. Untreated with low-grade diphtheria antitoxin. Killed on first day. Lipoidal materials may be seen to occupy practically the entire cortex. **Fig. 21.** Adrenal gland of rabbit with septicaemia. Receiving low-grade diphtheria antitoxin subcutaneously. Killed on first day. Lipoids may be seen to occupy only a small portion of the cortex. **Fig. 22.** Adrenal gland of rabbit with septicaemia. Untreated with low-grade diphtheria antitoxin. Killed on second day. The cortex is seen to contain still a fair amount of lipoid. **Fig. 23.** Adrenal gland of rabbit with septicaemia. Receiving low-grade diphtheria antitoxin by mouth. Killed on second day. Lipoids occupy much smaller amount of cortex than in Fig. 22.

were killed. Two rabbits died on the fourth day; the one had received serum by mouth, and the other had received no treatment.

One half of each of the adrenals was fixed in 10 p.c. formalin, and subsequently cut by the freezing method, and stained with Sudan III for lipoids. The second half was treated with osmium tetroxide, and subsequently sectioned and mounted.

First Day.

The adrenals of the rabbit receiving a subcutaneous injection of serum showed that lipoidal material was completely absent from one side of the gland, as evidenced by lack of staining with Sudan III. On the other side of the gland the lipoids were reduced to a small band situated near the periphery of the cortex. The zona glomerulosa and the outer region of the zona fasciculata in this portion of the gland possessed, however, no lipoids, and, apart from the small band mentioned, lipoids were absent from the remainder of the zona fasciculata and also the zona reticularis.

In the same gland the osmicated fat was concentrated chiefly in the outer fifth of the cortex, but this region was not sharply demarcated from the rest of the cortex.

The adrenal of the animal receiving no serum showed a very dense but thin rim of lipoidal material extending right round the gland, and extending through the cortex from this rim to the medulla were strands of cells containing similar large amounts of lipoids. The intervening cell areas contained also a certain amount of lipoid. The outer half of the cortex contained osmicated fat.

It was thus shown that, twenty-four hours after infection, a great efflux of lipoids had taken place with the serum-treated animal, while the adrenal of the untreated animal still possessed an excellent supply.

Second Day.

The adrenals of the untreated animal showed no lipoids in the outer region of the zona fasciculata or the zona glomerulosa. Cells containing a large amount of lipoid formed a ring around the adrenal near the periphery of the zona fasciculata. The cells of the middle portion of the zona fasciculata and the zona reticularis contained a moderate supply of lipoids, but none was found in the inner region of the zona reticularis.

Osmicated fat was found to occur almost entirely in the zona glomerulosa and the outer zona fasciculata.

The adrenals of the rabbit receiving serum by mouth showed a narrow band of lipoidal material in the extreme outer region of the zona fasciculata. The remainder of this zone and the zona reticularis contained none. The osmicated fat occupied the same situation as the lipoids.

On the second day, therefore, the serum-treated animal still possessed less lipoidal material than the control.

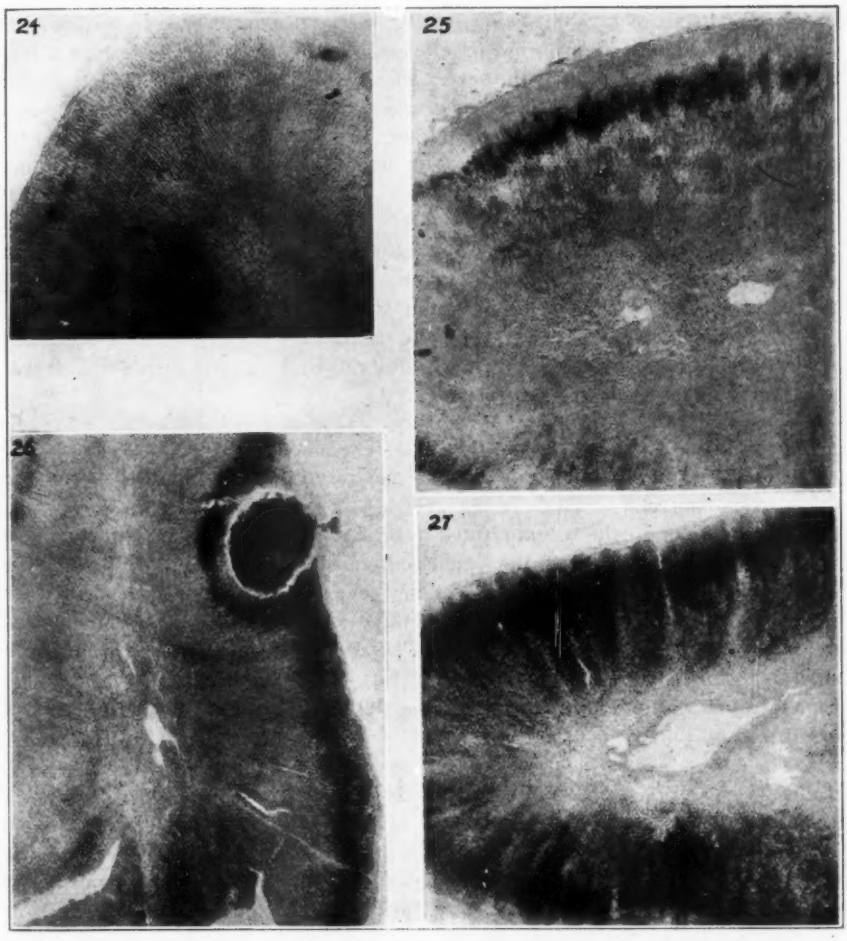

Fig. 24. Adrenal gland of rabbit with septicaemia. Receiving low-grade diphtheria antitoxin by mouth. Killed on third day. Only a very small amount of lipoid remaining. **Fig. 25.** Adrenal gland of rabbit with septicaemia. Receiving low-grade diphtheria antitoxin by mouth. Killed on third day. Only a thin band of lipoids present in the cortex. **Fig. 26.** Adrenal gland of rabbit which had died as a result of septicaemia. Untreated with low-grade diphtheria antitoxin. Almost one-quarter of the cortex contain lipoids. **Fig. 27.** Adrenal gland of rabbit which had died as a result of septicaemia. Treated with low-grade diphtheria antitoxin. Lipoids may be seen to occupy most of the cortex.

Third Day.

The adrenals of a rabbit being treated with daily doses of serum by mouth and killed on the third day were practically bare of lipoids. There were a few patches in the outer region of the cortex which gave a slight reaction with Sudan III, but the rest of the gland gave no reaction at all.

There was still, however, a considerable proportion of osmicated fat, aggregated chiefly in the peripheral portion of the cortex, and least of all in the zona reticularis.

The other rabbit killed on the third day was one receiving serum subcutaneously. There was a small, peripherally situated, not very deeply-staining, band of lipoids with a few cords of lipoid containing cells extending towards the medulla. Most of the cortex, however, contained no lipoids.

The zona glomerulosa and the outer portion of the zona fasciculata contained a very large amount of osmicated fat. In the remainder of the cortex the fat was of a much higher degree of saturation.

Fourth Day.

On this day two animals died. One had received serum by mouth and the other was a control.

The adrenals in the serum-treated animal contained what was practically a full load of lipoids, although the lipoid content of the cells thinned out towards the medulla. Only about half the medullary cells showed collapse. Osmicated fat in this gland was restricted to the outer third of the cortex only. The extreme outer portion of the zona fasciculata and the zona glomerulosa contained a considerable quantity of highly-unsaturated fat, the remainder of the osmiophilic zone staining a brownish colour, indicating the presence of a fat of a high degree of saturation.

The adrenals of the control animal showed a cortex, including the zona glomerulosa, containing a large supply of lipoids. Practically the whole of the cortex contained varying quantities of osmicated fat also, and the medullary cells showed a patchwork of light and dark cells (with the osmic acid stain). The dark cells were due to the blackening of adrenalin granules with the osmic acid. Some of the cortical cells showed a deep blackening, and in others it was only brownish or greyish, indicating the more saturated nature of the fat contained therein. The zona glomerulosa cells were also packed with unsaturated fat.

The results of these experiments with rabbits, therefore, appear to show that something is contained in low-grade diphtheria antitoxin administered either by mouth or subcutaneously, which possesses what one might call a discharging effect on the lipoids of the adrenal cortex—a corticotropic or lipotropic substance. In the case of the rabbits which died as a result of the infection, the adrenals of both control and experimental animals contained almost a full load of lipoids; this apparently enigmatical result is referred to again in the general discussion.

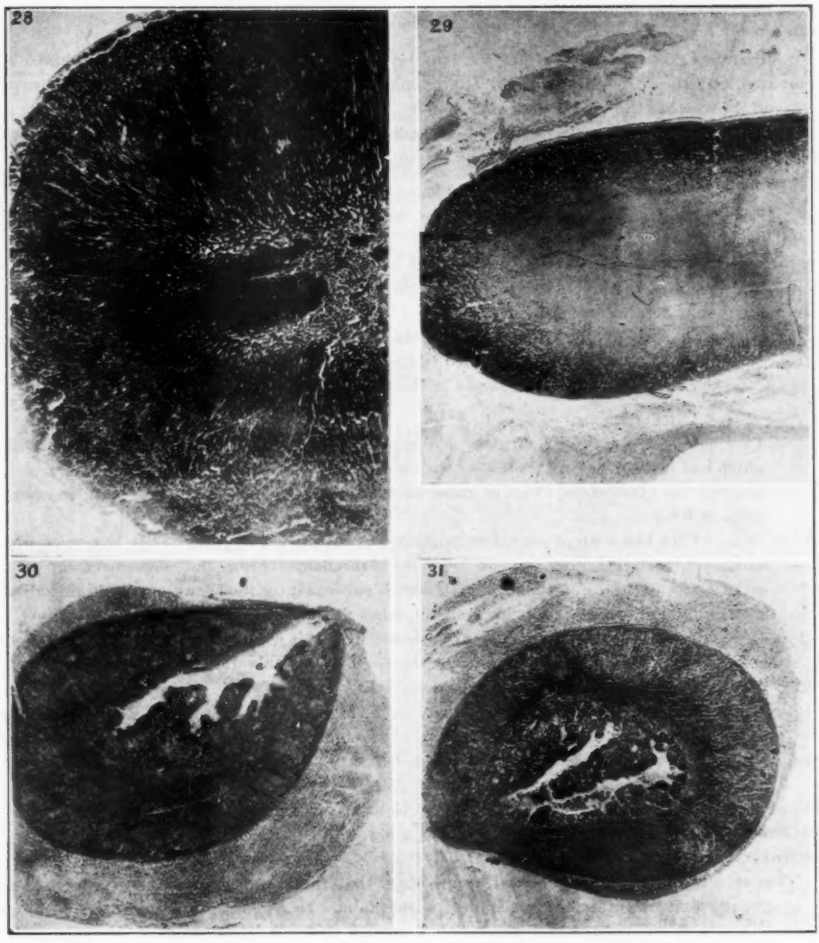

Fig. 28. The adrenal gland of a rabbit which died of septicaemia. Untreated with low-grade diphtheria antitoxin. Osmicated (unsaturated) fat may be seen to occupy the main width of the cortex. Compare Fig. 26, which is portion of the same gland stained to show lipoids. **Fig. 29.** The adrenal gland of a rabbit with septicaemia. Killed on second day. Receiving low-grade diphtheria antitoxin by mouth. Osmicated (unsaturated) fat may be seen to occupy principally the outer half of the cortex. Compare with Fig. 23, which is portion of the same gland stained to demonstrate lipoids. **Fig. 30.** Adrenal gland of mouse with septicaemia. Numerous granules of melanin may be seen in the cortex and medulla. **Fig. 31.** Adrenal gland of mouse with septicaemia. The "X zone" (Howard Miller, 1927) may be seen to be a centre of haemorrhage.

Experiments with Mice.

Experiment 1.

Mice were given serum in water for two days, and their adrenals were then examined and compared with the adrenals of mice of approximately the same weight and size, in an endeavour to ascertain whether the serum alone would affect the adrenal.

No difference could be observed between the lipoids in the glands of the treated and untreated animals.

Experiment 2.

Mice receiving serum, and untreated mice, were given intraperitoneal injections of a streptococcal suspension.

Of the untreated mice, one died in under 48 hours, and showed an adrenal completely devoid of lipoids. Two died in three days, and one showed an adrenal with no lipoids and the other an adrenal with only a faint trace.

Of the animals receiving serum by mouth, one died in under 48 hours, and showed an adrenal devoid of lipoids. Of two others which died in two days, one showed a narrow, peripheral rim of lipoids, and the other showed a little lipoid in the zona reticularis.

Experiment 3.

(*a*) 0·1 c.c. of a suspension of virulent streptococci was injected intraperitoneally into mice which had been given oral treatment with serum for forty-eight hours before the commencement of the experiment. Two of these animals died, and the adrenals of both showed no trace of lipoids.

(*b*) 0·15 cc. of the same streptococcal suspension was injected intraperitoneally into mice which had been receiving serum per os for forty-eight hours before the commencement of the experiment. Of two of these animals which subsequently died, one possessed an adrenal showing a slight trace of lipoids, and the other possessed no trace.

(*c*) 0·15 cc. of the original streptococcal suspension was injected intraperitoneally into mice which had had no previous serum treatment. Two animals died, and showed no trace of lipoids in their adrenals.

(*d*) 0·1 c.c. of the above suspension was injected intraperitoneally into mice which were given serum per os during the infection. Two of these animals died, and both showed adrenals with lipoids in the outer half of the adrenal.

These results support Cramer's statement that the previous work on this subject may be summarized by saying that "experimental infections so virulent or so massive that they lead to death in a few days produce, if the infection is allowed to run its course, a disappearance of the cortical lipoids . . .".

The results of the experiments with mice show that the low-grade diphtheria antitoxin has no apparent effect on either the resistance of the animal to the streptococcal infection or the adrenal lipoids. Now diphtheria toxin in large enough doses is lethal to most animals, and it appears to have a particular predilection for the adrenal gland, causing rapid exhaustion of the cortical lipoid and pathological inflammatory conditions in the cells. Cramer's statement, therefore (p. 119), that "a dose of diphtheria toxin which would kill one hundred guinea pigs can be injected into a mouse without producing obvious effects in the animals, and what is equally remarkable, without producing any change in their suprarenals", is of great significance, and the antitoxin apparently is also without action on the mouse or its adrenal gland. This would then perhaps explain the enigmatical results obtained from the mouse experiments. The conclusion is that mice are entirely unsuitable for experimental work in connection with diphtheria toxin or antitoxin. This immunity to diphtheria toxin in mice is all the more extraordinary when one remembers that the mouse and also its adrenal gland are susceptible to the toxins of most other bacteria.

Summary of Changes in Lipoids and Unsaturated Fat.

	Killed on First Day.		Killed on Second Day.		Killed on Third Day.			Died on Fourth Day.
	Control.	Serum subcutaneously.	Control.	Serum by mouth.	Serum subcutaneously.	Serum by mouth.	Serum by mouth.	Control.
SUDAN III STAINING MATERIAL (LIPOIDS)	Dense rim of lipoids in periphery of gland, rest of cortex contained high proportion.	Lipoids absent from one half of gland. In other half lipoids reduced to small band near periphery of cortex.	Peripheral ring of lipoids. Remainder of cortex contained none.	Narrow peripheral band of lipoids. Remainder of cortex contained none.	Thin, weakly staining peripheral band of lipoids. Most of the cortex contained no lipoids.	Practically no lipoids.	Practically a full load of lipoids.	Practically a full load of lipoids.
OSMIOPHILIC SUBSTANCE (UNSATURATED FATS)	Outer half of cortex.	Chiefly in outer fifth of cortex.	In zona glomerulosa and outer zona fasciculata.	Same as above.	In zona glomerulosa and outer zona fasciculata.	Considerable amount still in gland aggregated chiefly in peripheral region of cortex. Least in zona reticularis.	Restricted to outer third of cortex.	Contained in major portion of cortex.

HISTOLOGICAL CHANGES IN THE ADRENAL GLANDS IN SEPTICAEMIA.

(1) Rabbit Adrenals.

On the first day the adrenals of the animal which had been treated with serum showed evidence of congestion, haemorrhage, and hyperaemia, the last being particularly noticeable in the zona reticularis. The cytoplasm of practically all the cells in the various zones had become much more basophilic. Extensive damage to the gland had not yet, however, had time to take place, although even in the zona glomerulosa a few cells were observed with a contracted darkly-staining nucleus. In portions of the zona fasciculata there was contraction of the cytoplasm of groups of cells leaving wide interfascicular spaces. The cytoplasm of some of the cells showed signs of degeneration. The cells of the inner part of the zona fasciculata contained a large amount of lipochrome pigment, which was also found in quantity in some of the capillaries, presumably subsequent to breakdown of some of the cells.

In the zona reticularis cell breakdown was much more apparent. Of course it must be remembered, as Hoerr (1931) mentions, that the zona reticularis is

normally a region of cell breakdown. Nuclear pycnosis was very common in this region, and free pycnotic nuclei could be seen lying amongst the cells. In some of the cells observed in this region the nucleus was packed with melanin pigment, although no melanin could be observed in the cytoplasm. Many lymphocytes were observed in the widely-dilated sinuses of the zona reticularis, but they appeared to be adherent to the walls. Some cells also showed the presence of nuclear fragments, and cells with nuclei in all stages of contraction and breakdown were found. Most of the medullary cells were in an exhausted condition.

In the untreated animal killed on the second day, some nuclei of the zona fasciculata were found to be greatly hypertrophied and vesicular, others were contracted and pycnotic. Very few nuclei in mitosis could be seen, and breakdown of the cytoplasmic body was a common phenomenon; capillary congestion was again noticeable, together with small intracellular haemorrhages. A very large number of nuclei were contracted and deeply stained. Aggregation of the chromatin of the nuclei into granules and the subsequent breakdown of the nuclear membrane and liberation of the granules through the cell constituted a common method of nuclear disintegration. Subsequent breakdown of the cell wall and liberation of these pycnotic fragments followed.

In the adrenals of the rabbit receiving serum treatment there appeared to be a peripheral dilatation of capillaries; and in the inner regions of the gland the capillary vessels seemed swollen and congested. Quite a number of the cells of the zona glomerulosa appeared to be in a state of active mitosis.

The cells of the outer zona fasciculata exhibited large vesicular nuclei. The cells of the inner half of the zona fasciculata possessed nuclei in which the vesicular nature was lost. The diffusely staining chromatin of the vesicular form was now aggregated into small deeply-staining karyosomes.

Deeper still, cells of the zona fasciculata and the zona reticularis exhibited nuclei which were much more chromaphilic, and the chromatin was distributed diffusely. In the zona reticularis a very large number of these nuclei showed progressive changes into a typical pycnosis.

The medullary cells were in the exhausted condition described by Cramer, and a number of the cell nuclei were pycnotic.

On the third day a great deal of lymphocytic infiltration was seen in the adrenal of the rabbit receiving serum by mouth. The cortex also showed lateral thinning of the cords of cells in the zona fasciculata and condensation of the chromatin of the nuclei into large nucleoli which almost completely filled the nucleus. The lymphocytic infiltration was most common in the region of the zona reticularis. In this zone also, broken-down cells could be seen in the inter-cellular spaces. Similar cells were seen in the medullary sinuses together with large numbers of lymphocytes.

The adrenals of the rabbit receiving serum subcutaneously showed dilatation

of the cortical capillaries with a tendency for the lymphocytes to adhere to the capillary walls. Very little pycnosis could be observed in the outer region of the zona fasciculata, but the inner region of this zone and also the whole of the zona

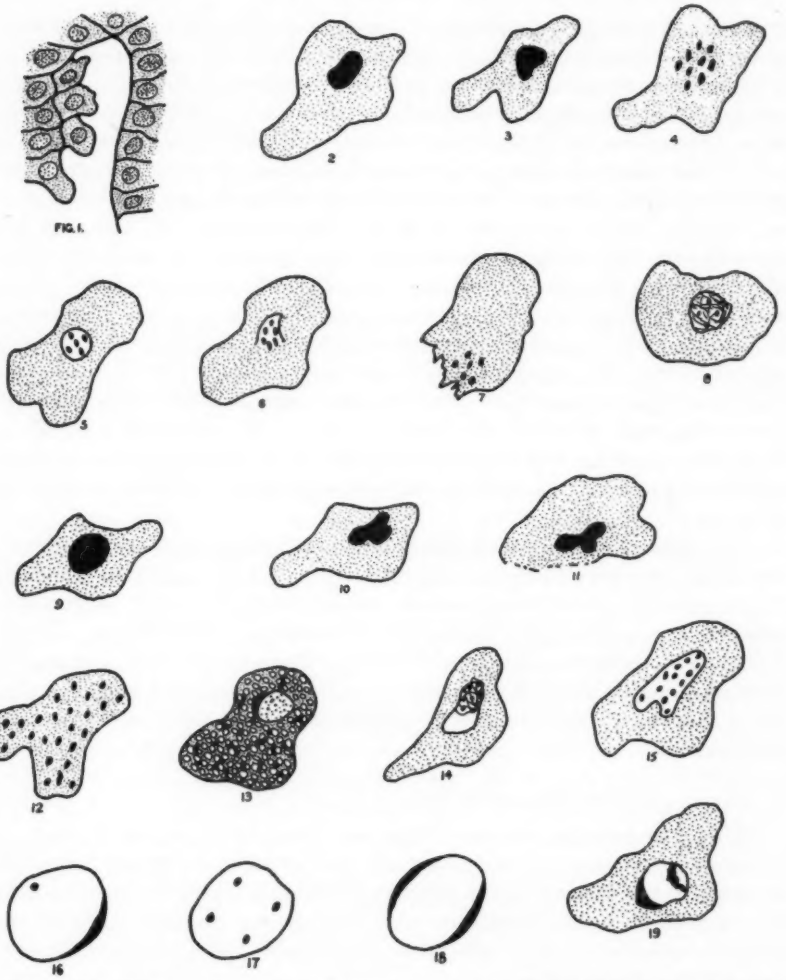

Fig. 1. Showing widening of interfascicular spaces in adrenal gland of animal with septicaemia. **Figs. 2–12.** Various stages of nuclear pycnosis and cell breakdown in adrenal gland of animal with septicaemia. **Fig. 13.** Showing fragments of Golgi apparatus extending through the lipoid droplets of a cortical cell of adrenal gland (animal with septicaemia). **Figs. 14, 15, and 19.** Further stages of nuclear disorganization. **Figs. 16, 17, and 18.** Distribution of chromatin-like material in red blood cells of mouse affected with septicaemia.

reticularis showed a distinct lymphocytic infiltration. Numerous small haemorrhages could be seen in the latter region.

Adrenals of Animals in the Pre-mortal State.

The adrenals of the animal receiving no serum showed a number of the medullary cells with melanin pigment in the nucleus, but with none in the cytoplasm. All the sinuses were congested with red blood corpuscles. A number of red blood cells contained granules of a basophilic material which appeared to be chromatin. In many cases portion of the rim of the corpuscle was found to be basophilic.

In these adrenals some preparations were made in an endeavour to demonstrate the Golgi apparatus. In some of the zona glomerulosa cells this apparatus was reticular, but in most it had broken up into granules. All the zona fasciculata cells and those of the zona reticularis also, contained an atypical apparatus. A diffuse black area in the cytoplasm in some cells represented the remaining remnant of the Golgi apparatus. In quite a number of the cells there appeared to be a very large number of granules resulting from the fragmentation of the Golgi apparatus, with also apparently some increase in amount of Golgi material, indicating a slight hypertrophy. This breaking-down and hypertrophy of the apparatus supports the belief that hyperactivity of the apparatus is required for the production of the cortical hormone, which is undoubtedly being produced to make good that which has been excreted into the blood stream as a result of the infection.

The adrenals of the animal receiving serum by mouth showed a marked degree of lymphocytic infiltration and quite a large number of pycnotic nuclei were also present. In the medullary sinuses, cell débris was present. Large masses of melanin could be seen in the cytoplasm of a number of cortical cells.

The serum appears, therefore, to have no effect on the histological reaction of of the gland to the infection, and there is no increase in mitotic activity. The action of the serum on the adrenal is apparently only in the direction of lipoidal excretion.

The Histology of the Adrenals in Mice.

The general results were similar to those observed in rabbits, but very striking haemorrhages into the zona reticularis were seen in some glands. Some of the cortical, and less commonly, the medullary cells showed the presence of chromophilic material in the cytoplasm staining with the same intensity as the chromatin of the nucleus. In one cell a mass of chromatin was apparently about to be extruded from the nucleus.

Lymphocytic infiltration was common, also the presence of masses of melanin in the nuclei. In one specimen, of eighty nuclei visible in a single high-power field, about thirty contained extensive deposits of melanin. In this specimen both the nucleus and cytoplasm of the medullary cells contained fine granules of melanin.

Infiltrating lymphocytes in this region and in the zona reticularis also contained intranuclear masses of melanin.

DISCUSSION.

The experiments with rabbits indicated the presence of some active principle in low-grade antitoxic serum, affecting the adrenal cortex. It was at first suspected that this active substance might have been the adrenal cortical hormone. It is known that diphtheria toxin causes an exhaustion of the lipoids of the adrenal gland which contain the cortical hormone, and therefore the serum of an animal which has had a series of injections of the disease might be expected to contain a higher proportion of these lipoids and consequently of the adrenal cortical hormone than the serum of an ordinary animal.

The operation of bilateral adrenalectomy was then performed on a series of six rats, and half the number were given oral post-operative treatment with the antitoxic serum, the other half serving as controls. Since an approximately equal number of rats in both control group and experimental group died from adrenal deficiency, it was concluded that, if the cortical hormone were present in the serum, it was present in too small an amount to prevent death from cortical deficiency in adrenalectomized rats.

The active substance is therefore as yet unknown. What, then, is the significance of the lipoidal changes of the adrenal produced by this postulated active substance of the serum?

Either the injection of the serum or its administration per os causes practically complete discharge of the lipoids of the adrenal cortex. This shows that the substance is unlikely to be a protein, or loosely attached to a prosthetic protein group, because, if protein, it would be destroyed by the digestive juices, and if the active principle is in prosthetic linkage with a protein it would not be available on injection as it would not receive the preliminary digestion by the alimentary enzyme, which would free it from its prosthetic protein group.

Individual requirements vary, and in one rabbit which died the adrenal was found to possess practically a full load of lipoids; that is, it presumably required a greater amount of the serum to cause discharge of the lipoids than the other animals. Death followed as a result of this inability to discharge the adrenal lipoids into the blood stream.

Cramer (1928) considered that any infection which causes the death of the animal will cause complete exhaustion of the adrenal lipoids. That this is not so is shown by the fact that two rabbits died with practically a full load of lipoids in the adrenal cortex, although it must be admitted that practically all the mice which died showed no lipoids in the gland.

Deanesly (1931) has pointed out that she has seen animals die from infection while still retaining some lipoidal material in the gland, and conversely she has found lipoid to be absent from the adrenal in cases where an animal has survived an infection.

The first effect of an acute infection appears to be partial or complete inhibition of the secretory mechanism of the adrenal cortex, and the cortical hormone is not available to aid the tissues and the body as a whole in their fight against infection. The low grade diphtheria antitoxin therefore appears to possess a substance capable of releasing this inhibition.

It appears that the discharge of the adrenal lipoids is an essential part of the defence mechanism of the body. The usual conception was that once the adrenal cortex was exhausted of its lipoids the animal was likely to fall a victim to the disease. This work indicates that survival from the infection is dependent upon an early discharge of the lipoids of the adrenal cortex followed by an increase in the amount of fat stainable with osmium tetroxide from which the lipoids can be reconstructed. There appears little doubt that the efficacy of the lipoids of the gland is due to their content of cortical hormone. Assuming that the effect of the serum is due primarily to its stimulation of the adrenal cortical lipoids, a more rational treatment would be the injection of the cortical hormone into such cases of streptococcal septicaemia as would normally receive treatment with low-grade diphtheria antitoxin, or perhaps a combination of the two would be more efficacious. There is no evidence that the adrenal gland is the only organ stimulated by the serum, but we have definite evidence that the adrenal is capable of giving a functional response to the serum whether the latter is administered subcutaneously or per os. It is notoriously dangerous to interpret too extensively histological changes in terms of function, but the evidence obtained has justified the making of definite statements on this subject.

In view of the increased incidence of puerperal septicaemia at the present time, the results obtained in this work justify a large scale clinical test of the serum with or without simultaneous treatment with the adrenal cortical hormone.

SUMMARY.

1. Low-grade diphtheria antitoxin administered subcutaneously or per os tended to cause discharge of the adrenal cortical lipoids in rabbits suffering from streptococcal septicaemia.

2. This antitoxin had no observable effect on mice suffering from streptococcal septicaemia.

3. The septicaemia caused cellular destruction in the adrenal glands similar to that observed by other authors.

4. It is suggested that the discharge of the lipoids containing the cortical hormone from the adrenal cortex, is a vital part of the defense mechanism of the body, and that if sufficient discharge does not take place the animal is liable to fall a victim to the infection.

5. It is strongly urged that, in light of these results, a large scale clinical test be instituted to determine the value of this anti-toxic serum, with or without the adrenal cortical hormone, in the treatment of puerperal septicaemia.

ACKNOWLEDGMENTS.

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PLEURO-PNEUMONIA CONTAGIOSA BOUM: THE STAINING OF THE CAUSAL ORGANISM IN THE SPECIFIC LESIONS

by

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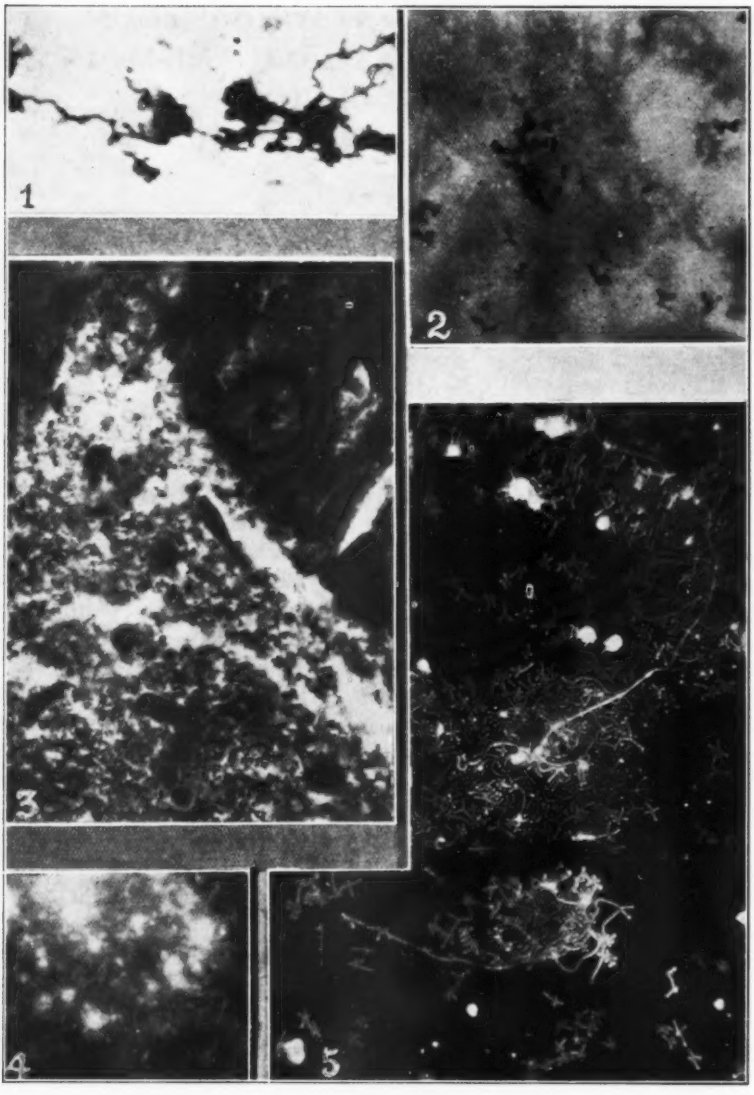
INTRODUCTION.

Ledingham (1933) and Turner (1933) have recently given adequate resumés of the earlier work on the morphology of the causal organism of pleuro-pneumonia of cattle. It was first cultivated in artificial media by Nocard and Roux (1898), and its morphology studied by Borrel and his colleagues (1910), but no one has succeeded in demonstrating it in microscopical sections of infected tissues. If this could be done it would strengthen the generally accepted claim that Nocard and Roux's organism is the causal organism of pleuro-pneumonia, afford a direct means of diagnosis, and facilitate study of the pathogenesis.

Nevertheless, no serious attempt has previously been made in spite of the fact that Borrel and his colleagues showed that, at least in artificial culture, the organism could reach a length of several microns. Our own studies of the organism grown in V.F.-O.S. broth and examined in the living state by dark-ground illumination [(Turner (1933) and Turner, Campbell, and Dick (1935] have demonstrated that it progresses from a filter-passing stage (conidioid) about $0{\cdot}2\mu$ to $0{\cdot}4\mu$ in diameter to a mycelial stage (fig. 5) in which relatively enormous elaborately branched forms up to 190μ in length have been observed, before finally and completely reaching the filterable stage again. As all phases of the organism grown in artificial culture can be stained in smears by various methods (fig. 1), and as the mycelial phase can be demonstrated in lung lesions and pleuritic exudate by dark-ground examination (fig. 4), there seemed to be no reason why it should not be possible to stain it in sections.

Martzinowski (1911) had indeed claimed to demonstrate it by staining unfixed impression-preparations of the cut surface of lesions with Giemsa, but the forms described (cocci and coccobacilli), which led him to propose the name *Coccobacillus peripneumoniae*, have little resemblance to what we now know to be the usual morphology, and it appears that his claims are not tenable.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TECHNIQUE.

For the purpose of a histological study of the disease, similar portions of pleuro-pneumonia lesions at various stages were placed in the following fixatives: 10 p.c. formol-saline, formol-Müller, Regaud, absolute alcohol, Carnoy, Bouin, Schaudinn, Schaudinn plus 5 p.c. of acetic acid, Zenker, and a mixture of mercuric chloride and osmic acid. It was found that the mercuric fixatives, especially Zenker, are excellent for both the specific organism and the tissue elements (and are essential for certain histological staining methods), but, owing to the necessity for prolonged washing of the fixed tissues and the final removal of traces of mercury by treatment with iodine, they are a little troublesome for routine purposes. Bouin (a picro-formol-acetic mixture), which is almost equally good for fixing the organism, is more adapted for routine purposes, and a large variety of histological stains may be used in conjunction with it. Carnoy fixes the organism well but is not a satisfactory general tissue-fixative, and does not permit the subsequent use of certain desirable histological stains; and even absolute alcohol, although a poor tissue-fixative and hence unsuited for pathological studies, allows the organism to be well stained. On the other hand formol-saline, formol-Müller and Regaud (a formol-bichromate-acetic mixture) are quite useless and appear to destroy the organism entirely; for it cannot be subsequently stained even after mordanting such sections with Zenker or Bouin. The formalin thus appears to be the injurious substance, which is the more remarkable because Bouin acts so well; presumably its other constituents (picric and acetic acids) act as correctives.

Once appropriately fixed, the organism stains readily, but a little care is needed during the differentiation or dehydration of sections, for it is easily decolourized by excessive treatment. "Basic" dyes that have given good results are victoria blue, methylene blue, basic fuchsin, gentian violet, Giemsa, thionine, Heidenhain's iron-haematoxylin, and Mallory's phosphotungstic acid-haematoxylin; but to the practised eye it is still recognizable even when treated with the "acid" or counterstain dyes, eosin, chromotrop 2R, and picric acid.

Although a large variety of methods is available, and although the mercuric

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EXPLANATION OF FIGURES.

Fig. 1. Long-branching filament from 20-hr. culture in V.F.-O.S. broth. Fixed with osmic acid vapour, mordanted with Loeffler's ink, stained with ammonia oxalate-gentian violet. [× 2400.] **Fig. 2.** Part of enormously distended interlobular lymphatic vessel showing branching filaments. Bouin's fixative, Heidenhain's iron-haematoxylin stain. [×1800.] **Fig. 3.** Small bronchiole almost filled with enormous numbers of *Borrelomyces*, monocytic cells, and desquamated bronchiolar cells. Bouin's fixative, Mallory's phosphotungstic acid-haematoxylin. [× 900.]. **Fig. 4.** Pleuritic exudate from a natural case of pleuro-pneumonia. Dark-ground photograph of centrifugate, showing branching filaments surrounding the mass of leucocytic and other debris. [× 1800.] **Fig. 5.** 24-hr. culture in V.F.-O.S. broth, photographed by dark-ground illumination. Note branching filaments many tens of microns in length, some of which are fragmenting to give rise to the filter-passing conidioids. [× 1200.]

fixatives, especially Zenker, are slightly superior, we recommend for general purposes fixation in Bouin followed by the following adaptation of Mallory's excellent staining method.

(a) *Fixation:*

1. As soon after death as possible (1) fix thin pieces of tissue, about 2 to 3 mm. thick, in Bouin's fixative (2) for 24 hours.

2. 50 p.c. alcohol.	1 or 2 hours.
3. 70 p.c. alcohol.	24 hours.
4. 90 p.c. alcohol	24 hours.
5. Absolute alcohol	24 hours.
6. Chloroform and clove oil	24 hours.
7. Chloroform	24 hours.
8. Saturated solution of paraffin in chloroform	1 hour.

(b) *Imbedding:* Imbed in paraffin (preferably in a vacuum imbedder at 25 cm. Hg. for 3 hours).

(c) *Sectioning:* Cut sections very thin (preferably about 4μ thick).

(d) *Routine staining:* After fixing on slides and bringing to water, and without preliminary treatment with potassium permanganate and oxalic acid, stain in Mallory's phosphotungstic acid-haematoxylin for 18 to 24 hours.

Without washing, blot nearly dry, and quickly dehydrate with absolute alcohol.

Clear in xylol, and mount in balsam.

Staining reactions. The organism stains a deep blue and appears very distinctly, even under the low power, as thick tangled masses of the mycelial phase.

The tissues stain in the usual excellent polychromatic manner with phosphotungstic acid-haematoxylin, i.e. nuclei are blue and sharply stained; protoplasm and muscle, various shades of blue; mucin, reddish-yellow; cartilage, a deep rose-red; fibrin, deep blue; erythrocytes, a steel-blue.

A slight washing in tap water, followed by a quick differentiation in 95 p.c. alcohol before the dehydration, produces much better histological differentiation but the organism stains less intensely, and instead of the deep blue may assume a reddish-purple or reddish colour.

Even after fixation in absolute alcohol it is stained fairly well by the above method.

Under the high power or oil-immersion lenses the much branched, relatively long, but very delicate filaments are easily recognizable, and resemble closely those described by us as a result of dark-ground observation of lung juice, pleuritic exudate, and cultures. Very occasionally asterodiscules may be found. The masses of micro-organisms, easily distinguishable on morphological grounds from fibrin and cellular debris, occur even in very early lesions in the terminal bronchioles (fig. 6); in more advanced lesions, they are seen in the distended lymphatic vessels of the subpleural and interlobular supporting tissue (fig. 8), in the alveoli (fig. 7), and bronchioles (fig. 3).

In the bronchial and mediastinal lymphatic glands of natural cases of pleuro-pneumonia, and in the adjacent lymphatic glands such as the prescapular of cattle subcutaneously inoculated, the organism occurs in large masses in the larger lymphatic vessels near the hilus.

Although generally it retains its known slender width in most sites, yet in certain parts of the sections it may appear much wider. In very large clumps near the edges of the sections, frequently

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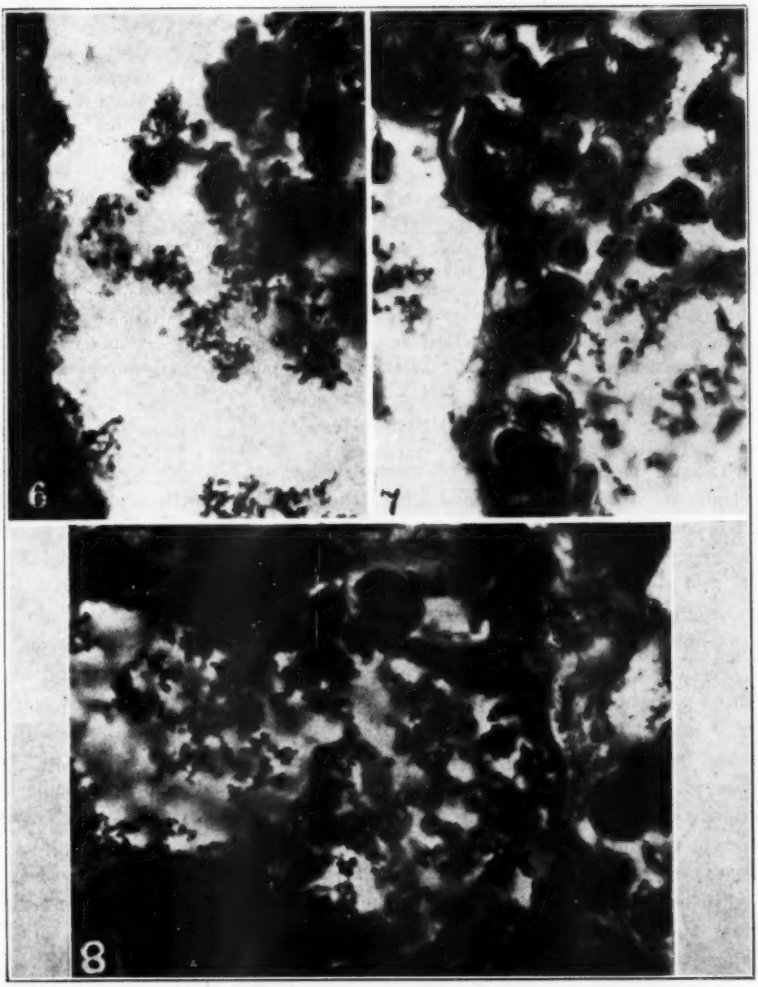
1 No deterioration is appreciable even if fixation is delayed for as long as six hours after death.

2 *Composition:* Formalin, 25; saturated aqueous solution of picric acid, 75; glacial acetic acid, 5.



EXPLANATION OF FIGURES.

Fig. 6. Terminal bronchiole, acute pleuro-pneumonia. Note the large masses of the mycelial phase of the *Borrelomyces*, and the monocytic cells. Bouin's fixative, Mallory's phosphotungstic acid-haematoxylin. [× 1200.] **Fig. 7.** Two neighbouring alveoli containing large numbers of the mycelial phase and monocytic cells. Bouin's fixative, Mallory's phosphotungstic acid-haematoxylin. [× 1200.] **Fig. 8.** Greatly distended subpleural lymphatic vessel containing large numbers of the mycelial phase. Zenker's fixative, Mallory's phosphotungstic acid-haematoxylin. [× 1200.]

only those organisms in the outer zone stain deeply, while those within the clumps exhibit instead a reddish-violet colour.

(e) *Other staining methods:*

(i) Heidenhain's iron-haematoxylin method, with careful differentiation, gives very sharp and delicate results (fig. 2), but is not a good stain for general pathological histology.

(ii) Good results are obtained (only after Zenker fixation) with Goodpasture's carbol-anilin-fuchsin followed by Loeffler's methylene blue (for details see Wadsworth, 1927). The organism usually takes the fuchsin, and thus appears red; the erythrocytes are vivid red, connective-tissue fibres pink, and the other tissue elements various shades of blue. If the staining is carefully performed it is possible to stain the organism a deep blue.

(iii) The azan method, after Zenker fixation, stains the organism blue or reddish purple, depending upon the degree of differentiation.

(iv) Eosin and methylene blue, after Zenker fixation, stains the organism blue, and the tissues in the usual excellent manner, but needs great care in the differentiation.

(v) Giemsa's stain is excellent for demonstrating the organism in sections. It is, however, easy to over-decolourize the filaments, and therefore a little troublesome for routine use.

DISCUSSION.

Although the pleuro-pneumonia organism has not hitherto been demonstrated in sections of pathological material, and although some excellent and painstaking work has consequently been devoted to attempting to deduce its presence in lesions and interpret the pathogenesis by the histological reactions [notably by Ziegler (1921) and Seifried (1926)], the fact remains that it is really a comparatively easy micro-organism to stain; indeed, *once it is appropriately fixed, it will stain distinctly enough by many methods*: everything depends upon the fixation. We have recently succeeded in demonstrating it quite easily in some faded haematoxylin-van Gieson sections of pleuro-pneumonia lung prepared over 20 years ago by Dr. J. A. Gilruth. After removal of the coverslips the sections, which fortunately had been fixed in sublimate-alcohol, were restained without further treatment in phosphotungstic acid-haematoxylin, and yielded some of the best examples of the delicate, branching organism that we have seen. Others restained by Giemsa were also excellent.

It therefore appears remarkable that the organism has not hitherto been described in sections. One explanation is the unfortunate coincidence that formalin solutions and formol-saline, which are the most usual routine fixatives especially for lung tissue, and which were used exclusively by Ziegler and Seifried, happen to be the fixatives after which it cannot be stained; the other is very probably the widespread, almost traditional view that it belongs to the "filterable viruses", and would therefore be difficult to demonstrate. Such is the power of the association of ideas that probably very few workers have considered it profitable to attempt to stain it, and, when it has been unwittingly stained, have failed to recognize it, owing to an insufficient acquaintance with its typical morphology.

Its ready demonstration in extra-cellular situations as described above and

its general morphological resemblance to the Gram-negative streptothrices should be one more reason for rejecting it irrevocably from the filterable viruses *sensu stricto*. In an article [3] based on a study of morphology and life-cycles, we recommend the erection of a new family *Borrelomycetales* in the class *Schizomycetes*, with a single order *Borrelomycetaceae* and a single genus *Borrelomyces* to contain it and the closely-related organism of agalactia of goats. According to this recommendation, the organism of pleuro-pneumonia is known as *Borrelomyces peripneumoniae*.

A study of the method of infection and the pathogenesis of the disease, based upon the above technique, is at present being carried out.

The presence of such enormous numbers of the organism, even in very young lesions, should be further evidence of its causal relationship to the disease, and the ability to demonstrate it in material collected after death should prove a useful addition to diagnostic procedures.

SUMMARY.

1. Nocard and Roux's organism of pleuro-pneumonia can be readily demonstrated in sections of pathological tissues by a variety of staining methods, provided it is suitably fixed.

2. Formol-saline, formol-Müller or Regaud, are absolutely contra-indicated.

3. Suitable fixatives are the mercuric-chloride group (especially Zenker), Bouin (picro-formol-acetic), absolute alcohol, and Carnoy.

4. The recommended technique for routine purposes is fixation in Bouin's solution followed by the staining of paraffin sections by Mallory's phosphotungstic acid-haematoxylin.

5. The organism is present in infected lungs mostly in the relatively enormous mycelial phase.

6. It is considered that its easy demonstration in extra-cellular situations is another reason for its exclusion from the filterable viruses *sensu stricto*.

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A YELLOW BACTERIUM ASSOCIATED WITH "HALO BLIGHT" OF BEANS

by

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At present, at least three distinct bacterial diseases of the bean (*Phaseolus vulgaris*) are generally accepted to exist, and there are possibly others (Burkholder 1930), though they have not received the same general recognition. Two of these diseases, viz. "Bacterial Blight", caused by *Phytomonas phaseoli* (E. F. Smith) S.A.B., and "Halo Blight", caused by *Phytomonas medicaginis phaseolicola* Burk., produce similar symptoms. Water-soaked spots on the leaves are characteristic of both diseases, though in the case of "Halo Blight" they are often surrounded by a yellowish halo. The organisms causing these two diseases, however, are readily distinguishable by cultural methods.

In Victoria, "Halo Blight" has become by far the most destructive and widespread disease of the French bean. While making isolations from diseased material by the usual methods, we have frequently encountered a rapid-growing yellow bacterium on the isolation plates. Colonies of this organism, from diseased stems, leaves, or pods, sometimes develop alone as apparently pure cultures, and at other times as mixtures with the slower-growing colonies of *P. medicaginis phaseolicola*. It was the frequency of the development of these colonies from seeds suspected to be infected with *P. medicaginis phaseolicola* that first attracted our attention to the matter dealt with here.

The seed used for these isolations was principally of the white-seeded "Magnum Bonum" variety. Such infected seed may show well-defined lesions due to the accumulation of dense masses of bacteria in the third layer of the seed coat. As the number of isolations from this seed increased and the results accumulated, we were able to correlate the probable isolation of the yellow bacterium with the colour of the seed lesion. Where the lesions were antimony-yellow according to Ridgway's standard (1912) the yellow organism was isolated consistently, and where, by the same standard, they were maize-yellow *P. medicaginis phaseolicola* could be expected. The frequency with which the yellow organism was isolated and the fact that it often developed as a pure culture on the isolation plate raised

the question of its possible pathogenicity. To test this point a number of inoculation experiments were conducted using pure cultures of the yellow organism. Bean plants grown in a greenhouse at different times of the year were inoculated with suspensions sprayed on the plants or introduced by needle puncture, but in all cases disease symptoms failed to appear. It is unlikely that this failure was due to unfavourable environmental conditions, because plants inoculated at the same time with *P. medicaginis phaseolicola*, and kept under the same conditions, invariably developed symptoms of disease. We concluded then that the yellow bacterium when introduced as a pure culture into bean plants was non-pathogenic.

A yellow bacterium similar to that associated with this "Halo Blight" disease has been isolated quite frequently from "Mulberry Blight" lesions, and on occasions from lesions on gladiolus, stock, oleander, and soya bean.

DESCRIPTION OF THE YELLOW BACTERIUM.

The following description applies in particular to two isolates whose cultural characteristics, so far as examined, were indistinguishable. One of the isolates was from a bean seed, the other from a "Mulberry Blight" stem lesion of *Morus rubra*.

Morphology and staining. A small, motile, Gram negative rod, approximately $0{\cdot}8\mu \times 0{\cdot}5\mu$.

Beef extract agar. A very rapid grower, colonies appearing within 24 hours at 27°C. At first they are white to pale yellow, and smooth, circular, moist, with an entire margin. In 2–3 days the colour of the colonies becomes deeper yellow.

Beef extract broth. A uniformly heavy growth within 24 hours, later becoming more abundant, with a yellow sediment.

Potato. On potato cylinders a good watery spreading growth develops in 24 hours.

Fermi's solution. Abundant growth occurs, the medium becoming strontian yellow (Ridgway) after about 7 days. Later the solution becomes rather slimy.

Cohn's solution. No growth.

Litmus milk. The milk becomes slightly acid after 2 days, and later is decolourized. In 2–3 weeks it is a yellow-ochre colour (Ridgway). There is a slight peptonization.

Gelatine stab. Gelatine is liquefied in about 7 days.

Sugar fermentation. With sugar peptone media, acid but no gas is produced within 24 hours from glucose and saccharose, but not from lactose.

Starch agar. There is no evidence of diastasic action.

Nitrate reduction. There is strong reduction of nitrates within 24 hours.

Peptone water. There is no production of indol.

REVIEW OF LITERATURE.

The isolation of yellow non-pathogenic bacteria from diseased plants has been reported quite frequently, but often only as a passing reference. Thus Smith and Townsend (1907) mention a yellow bacterium developing with *B. tumefaciens* on isolation plates made from Crown galls. Manns (1909), and later Elliot (1920), report the isolation of yellow bacteria from the lesions of "Halo Blight" of oats. Manns believed that a yellow organism was jointly responsible with *P. avenae* in causing oat "Halo Blight". Bryan (1924) refers to a yellow bacterium associated with a bacterial leaf-spot of delphinium.

Rosen (1928) states that "a yellow schizomycete, entirely unrelated to the fireblight pathogen (*B. amylovorus*), is with astounding frequency to be found following up the work of the primary

parasite''. The yellow organism was found to occur not only in young, succulent twigs, but also in green pear fruit. He considered that this secondary invader might serve to limit the amount of host tissue invaded by the pathogen.

Burkholder and Guterman (1932) report the frequent association of a yellow bacterium with *P. hederae* in a leaf spot disease of the Ivy (*Hedera helix*), and bring forward evidence to show that the presence of the yellow organism accelerates the rate of development of the leaf spot caused primarily by *P. hederae*. More recently, White and McCulloch (1934) working with the same disease, isolated a number of yellow bacteria from old spots on the foliage. They found that one of the cultures of the ten lots of bacteria, when mixed with *B. hederae* and reinoculated into the Ivy, produced a distinct increase in the size of the lesion; but when the other associated bacteria were used, the reverse occurred, i.e. the infected spots were smaller than those produced by the host tissue invaded by the pathogen.

Yellow bacteria have been reported quite frequently in connection with ''Mulberry Blight''. Indeed, in some of the earlier Italian work on this disease, yellow bacteria present in the ''Mulberry Blight'' lesions appear to have been confused with the true causal agent. E. F. Smith (1920) and Wormald (1924), among others, have obtained yellow organisms from ''blight'' lesions, but concluded from inoculation experiments that they were not the cause of the disease. These workers confirmed the evidence of Boyer and Lambert that ''Mulberry Blight'' was caused by a slower growing bacterium that produced hyaline white colonies. Nosé (1933) recently claims to have shown that the yellow bacterium associated with *P. mori* in ''Mulberry Blight'' was antagonistic to the pathogen in culture media.

It is of further interest to note that Higgins (1930) mentions a yellow organism associated with ''Halo Blight'' lesions of the bean.

A consideration of this literature review has led us to examine the facts presented in the introduction in a little more detail. The remainder of this paper is devoted to the results of our investigation in this direction, and for convenience is dealt with under three headings.

EXPERIMENTAL.

(1) *The Intimacy of the Association of the Yellow Bacterium with the Host Plant.*

Although it is quite a common experience to find saprophytes following a pathogen as secondary invaders of diseased tissue, it seldom happens that the invader is so frequent and so uniformly of the one type as is the yellow bacterium now under consideration. We have found that the yellow bacterium may invade the bean plant and develop systematically in close association with the pathogen *P. medicaginis phaseolicola*, and may even reach the seed.

Experimental evidence on this point was secured when healthy bean plants were inoculated in the stem with mixed cultures of *P. medicaginis phaseolicola* and the yellow bacterium. After a period, isolations were made from the stem at different distances above the point of inoculation. The type of result obtained is illustrated in the following experiment.

On 16/12/33 a young bean plant was inoculated at the first leaf node with a mixed culture of *P. medicaginis phaseolicola* and the yellow bacterium. On 16/3/34 the plant showed symptoms of disease, but had survived to flower and set seed. On the same date isolations were made from the bacterial exudate at the point of inoculation, and also from the stem at points 2. 40, and 80 mm. respectively above the site of inoculation. The results of such isolations are shown in Table 1.

TABLE 1.

Isolations of P. MEDICAGINIS PHASEOLICOLA *and the Yellow Bacterium from a Diseased Bean Plant.*

Site from which the isolation was made.	Result.
Exudate at point of inoculation.	Pure culture of the yellow bacterium.
Stem, 2 mm. above point of inoculation.	Mixed culture, yellow colonies predominating.
Stem, 40 mm. above point of inoculation.	Mixed culture, colony types in about equal proportions.
Stem, 80 mm. above point of inoculation.	Mixed culture, colonies of *P. medicaginis phaseolicola* predominating.

Further evidence on the intimacy of this association is seen in the fact that the seed itself is invaded by the yellow bacterium. This was shown by the characteristic colour of lesions on the white seeds of the "Magnum Bonum" variety, and by the fact that in cross-sections bacteria could be observed in the third layer of the seed coat. Although it was not possible to say what organism was present in these sections, indirect evidence was secured by making isolations from the seeds. After disinfecting the surface of the seeds with mercuric chloride (0·01 p.c.) they were washed and broken up in sterile water. Subcultures then made from the extract proved the presence of the yellow bacterium in abundance. That the superficial disinfection was effective for the purposes intended was proved by the controls, where apparently healthy seed similarly treated yielded sterile plates. The seeds used for these isolations had been threshed from the pods, and it was not possible to determine whether the infection had taken place by way of the funiculus or through the micropyle, both of which, as Zaumeyer (1932) has shown, are means by which the seed may become infected.

(2) *The Effect of the Presence of the Yellow Bacterium on the "Halo Blight" Disease.*

The results of experiments to be described below show that a mixture of the yellow bacterium with *P. medicaginis phaseolicola* alters some of the symptoms of "Halo Blight" and modifies the rate of development of the disease.

The principal symptom-modification induced by the yellow organism is a brick-red colouration of the "grease spot" lesions on the pods. This reddening may develop over the whole lesion or be confined to the edge of the spot, and can be reproduced under experimental conditions. Pods inoculated with *P. medicaginis phaseolicola* alone developed typical water-soaked spots, but inoculations with mixed cultures produced brick-red spots similar in appearance to those described by Burkholder (1926). These brick-red lesions appeared in from two to three days, and from them the yellow organism was readily isolated, often as a pure culture. It is interesting to note that the lesions on English Ivy, from which Burkholder and Guterman isolated *P. hederae* and a yellow organism, were described as having reddish edges.

The antimony-yellow coloured lesions on "Magnum Bonum" seed, already

mentioned, have also been reproduced by using a mixed culture and needle-prick inoculation. The immature seeds were inoculated through the pods, and re-isolations from them yielded pure cultures of the yellow organism.

The effect, following inoculation with mixed cultures, on the rate of development of the disease, may be illustrated by reference to an experiment conducted in the greenhouse and commenced on 30th December, 1933.

Twenty-four healthy bean seedlings, three weeks old, were selected, divided into four groups of six plants each, and inoculated at the primary leaf node with the following cultures: (a) A 24-hours-old broth culture of the yellow bacterium. (b) A 24-hours-old broth culture of *P. medicaginis phaseolicola*. (c) A culture consisting of 24-hours-old broth cultures of the yellow bacterium and *P. medicaginis phaseolicola* mixed in equal proportions just prior to use. (d) Sterile water.

The plants were kept in a greenhouse till 17th January, 1934, when the following observations were made: All the plants of groups (a) and (d) were healthy. In group (b), two of the plants were dead and two showed advanced symptoms of wilting. In group (c) all the plants were infected, but in no case was the disease as advanced as that of any in group (b). Three plants showed early signs of wilting, and three showed no evidence of wilting.

Fig. 1 is a photograph of the two middle plants in groups (b) and (c), respectively, and reveals the average condition of plants in these groups on 17th January, 1934.

Re-isolations from plants in group (b) all yielded pure cultures of *P. medicaginis phaseolicola*, while plants in group (c) yielded either pure cultures of the yellow bacterium, or mixtures of the two organisms, or else pure cultures of *P. medicaginis phaseolicola*.

This experiment was repeated on 23th January, 1934, using five plants in each group, and similar results were obtained.

Figure 1. Photograph showing the effect of the use of a mixed inoculum. The two plants on the left were inoculated with a pure culture of *P. medicaginis phaseolicola*, and those on the right with a mixed culture of *P. medicaginis phaseolicola* and the yellow bacterium.

In other experiments inoculated plants have been allowed to grow as long as they would. In the case of seedlings inoculated with the mixture a number survived and produced seed, but in no case did the seedlings inoculated with pure cultures of *P. medicaginis phaseolicola* survive to produce seed.

Further information on the influence of the presence of the yellow bacterium on the disease was afforded by germination tests with "Magnum Bonum" seeds showing visible lesions.

Three lots of seed were selected from the one sample, viz.: (a) 16 seeds with antimony-yellow lesions (indicative of the presence of the yellow bacterium). 16 seeds with maize-yellow lesions (indicative of the presence of *P. medicaginis phaseolicola*). (c) 16 seeds with no lesions.

There was no significant difference in the actual size of the lesions in the two groups (a) and (b). All seeds were planted separately in pots and kept in the greenhouse, and the following results observed:

Six seeds of lot (a) germinated and all grew to maturity, producing healthy seed. Four of these plants appeared quite healthy, while two were infected, showing typical watersoaked spot lesions on the primary leaves. From these spots *P. medicaginis phaseolicola* mixed with the yellow bacterium was isolated.

Only one seed of lot (b) germinated, and the seedling died before the cotyledons had emerged above ground.

Thirteen seeds of lot (c) germinated, and all were healthy, remaining so until maturity.

The seeds that had lesions symptomatic of the presence of the yellow organism thus had a definitely higher rate of germination than had the seeds with lesions symptomatic of *P. medicaginis phaseolicola* alone. In most cases those seeds infected with the yellow bacterium were able to produce healthy plants.

From these results it is concluded that the presence of the yellow bacterium adversely affects the development of "Halo Blight" in beans.

In diseased plants the effect appears as a retardation in the rate of development of the disease. Where the yellow bacterium is present within the seed (and results already described suggest that this cannot occur in beans without infection by *P. medicaginis phaseolicola*), such seed may produce healthy plants, indicating thereby that the presence of yellow bacteria in these circumstances may completely inhibit the development of the pathogen.

(3) *The Specificity of the Association.*

The association of a yellow bacterium with *P. mori* in the lesions of blight on *Morus rubra* has already been mentioned. This yellow bacterium, mixed with *P. mori*, has been inoculated into mulberry stems. The results have shown that there exists a relationship between this yellow bacterium, *P. mori*, and the host which is quite comparable with that already described in the "Halo Blight" disease of beans.

It was also found that this yellow bacterium isolated from *Morus* when mixed with *P. medicaginis phaseolicola*, and inoculated into beans resulted in the development of brick-red coloured lesions indistinguishable from those produced by

the mixture of *P. medicaginis phaseolicola* and the yellow bacterium from the bean. Since no appreciable difference in cultural behaviour was found, and no other basis for their differentiation could be discovered, it was concluded that the same organism was associated with both diseases.

Repeated inoculation experiments have established the pathogenicity of *P. medicaginis phaseolicola* for the bean, while results from the inoculation of this organism into *Morus* have been uniformly negative. The same behaviour, *mutatis mutandis*, was proved for *P. mori*. Such specificity, however, does not hold for the yellow bacterium. Although it is itself non-pathogenic, it appears to be able to follow either *P. medicaginis phaseolicola* or *P. mori* in their respective hosts. Its capacities in this direction may, of course, be much wider.

SUMMARY.

A yellow bacterium whose cultural characteristics are described, has frequently been found associated with lesions of the "Halo Blight" disease of beans. Isolation plates from diseased material often yielded pure cultures of the yellow organism, but when inoculated as a pure culture into bean plants this organism produced no visible symptoms of disease.

Examinations have shown that the yellow organism, by closely following the pathogen *Phytomonas medicaginis phaseolicola*, may become systemically distributed within the host.

The presence of the yellow bacterium at the seat of infection may modify the symptoms of "Halo Blight". If the yellow bacterium is mixed with *Phytomonas medicaginis phaseolicola* at the time of inoculation then the rate of development of the disease is retarded and its ultimate severity is diminished.

A yellow bacterium isolated from "Mulberry Blight" lesions was found to be similar in morphology and physiology to the yellow bacterium isolated from bean plants. Furthermore, when mixed with *Phytomonas medicaginis phaseolicola* and inoculated into bean plants, it produced reactions similar to those obtained by inoculations with *Phytomonas medicaginis phaseolicola* and the yellow bacterium isolated from bean plants.

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THE DISTRIBUTION OF VITAMIN C IN LOWER ORGANISMS

by

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It has been shown in a previous paper (1935) that, according to the acid silver nitrate staining reaction, some reduced vitamin C occurs in every cell of the body. Some cells were found to contain only one or two granules, but the cells of certain glandular organs were observed to possess a very large number.

Giroud and Leblond (numerous papers during 1934) have obtained similar results, and have shown that reduced vitamin C could be observed in the cells of higher plants, particularly in those regions where the greatest amount of chlorophyll occurred.

In this investigation a number of lower forms of life were examined for the presence of reduced vitamin C. Previous work on the lower forms has been confined to observations by Giroud and Leblond on a specimen of Alga—*Zygnema*. The following have been examined for this work: *Chlamydomonas* (Protista), *Oscillatoria* (Cyanophyceae), *Scenidesmas* and *Spirogyra* (Chlorophyceae), Diatoms, Lichens, and a numer of fungi-species of *Fusarium, Ophiobolus, Coletrotrichum, Trichoderma, Helminthosporum, Rhizoctonis*, and *Trichoderma*. Cells of *Saccharomyces cerevisiae* (yeast) were also examined, as well as the mycelium of the saprophytic fungi *Mucor* and *Penicillium*, and specimens of the *Bacillus coli communis*.

In addition to these lower forms, the cells of certain organs of the tadpole in tissue culture were subjected to the silver nitrate staining test.

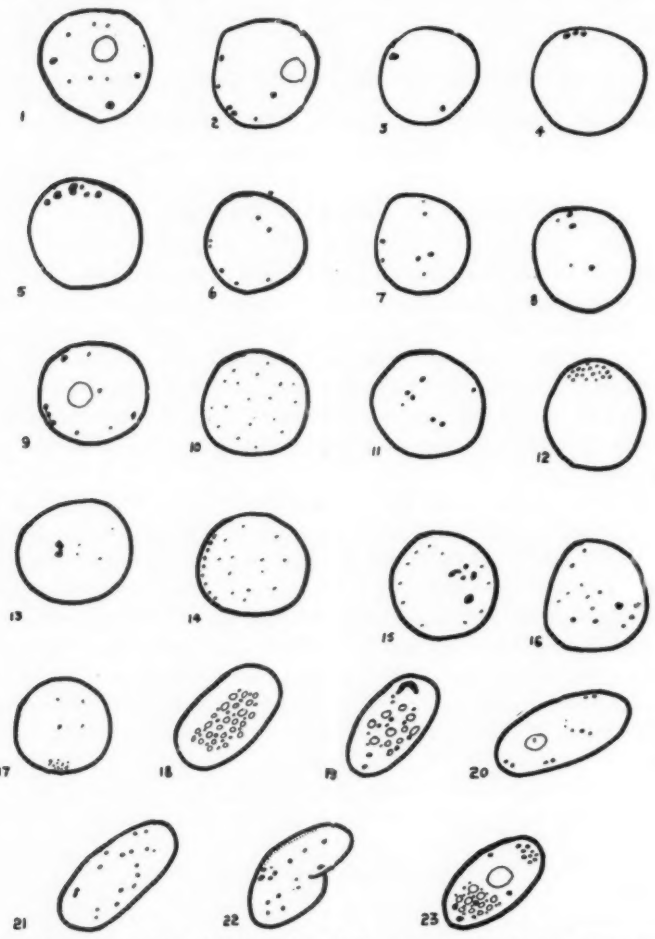
DISTRIBUTION OF VITAMIN C.

Chlamydomonas.

Whole specimens of *Chlamydomonas* were subjected to treatment with the vitamin C reagent (acetic acid—silver nitrate), but the presence of chloroplasts obscured the granules so formed and an accurate estimation as to the number of granules present was not possible. Large numbers of *Chlamydomonas* were therefore centrifuged, and then treated with the reagent for one hour. They were washed several times with distilled water, and then treated for an hour with an aqueous

solution of photographic "hypo". This was followed by further washings with distilled water. The organisms were then dehydrated, cleared in xylol, imbedded in wax, and cut in sections 2μ thick. Certain of the sections were subsequently stained, but some were examined without staining.

No constant form or localization of the resulting granules within the cell was obtained. In one specimen about nine granules could be seen—varying in size



Figs. 1–17 show the variety of distribution of the granules of reducing substance (vitamin C?) in the cytoplasm of different specimens of *Chlamydomonas*. **Figs. 18, 19, and 23.** Longitudinal sections of ciliates to show granules of reducing substance (vitamin C?) scattered among the vacuoles. **Figs. 20, 21, and 22.** The disposition of the granules in ciliates showing no vacuoles. In Fig. 22 a line of small granules may be seen running along portion of the periphery of the organism, giving the impression that they are basal granules of cilia.

from minute specks to quite large masses of argentophil material. In another, six large granules were observed to be scattered irregularly through the cell body. In a further specimen only two large granules could be observed, and in another three large granules were seen which were close together and apparently attached to the periphery of the organism. A further specimen exhibited a polar aggregation of argentophil granules, six of various sizes being clustered to one end of the cell body. Various other specimens showed eight or nine small granules scattered throughout the cytoplasm, but one could be seen to contain about twenty, quite small, granules, while another had some forty odd, with a tendency for a ring of granules to form round the periphery.

One specimen showed a complete aggregation of about twenty large granules at one pole, no other granules being present in the cell body.

Occasional cells were observed which possessed granules sticking to the outside of the cell—this would indicate the presence of small quantities of extra-cellular vitamin C. These specimens suggested the possibility that vitamin C was synthesized by the *Chlamydomonas* in excess and that the unused portion was secreted into the medium. Chemical tests of the water in which the *Chlamydomonas* had been growing, using the indophenol reduction method of Harris, showed that no detectable amount of reduced or oxidized vitamin C could be found in the medium. The conclusion is that, if any vitamin C is excreted by these organisms, it is either in too small a quantity to be detectable or it is rapidly destroyed by substances present in the medium.

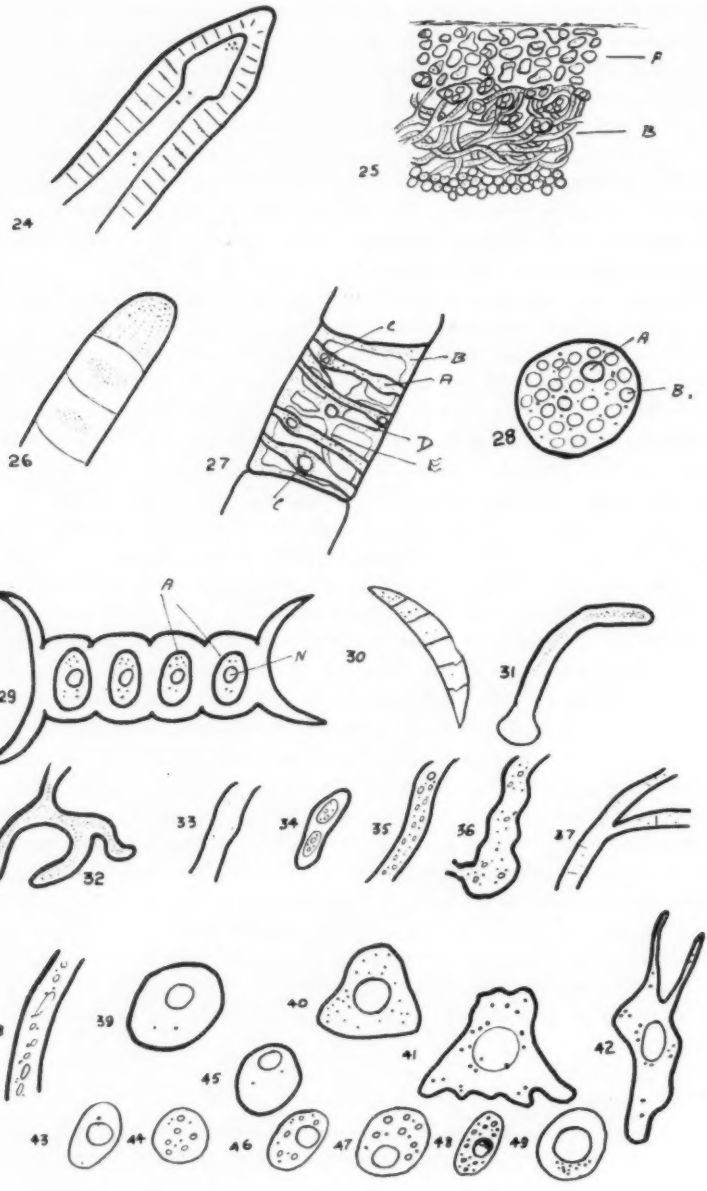
A number of ciliates were also treated in the same way as the *Chlamydomonas*. In ciliates which contained a number of small vacuoles the major portion of the reduced vitamin C was scattered in the form of small granules between the vacuoles. Any granules of vitamin occurring outside this vacuolar zone were very large. In one ciliate examined a number of small granules were scattered among vacuoles on one side of the nucleus and nine large granules were aggregated close together on the opposite side. There were no vacuoles on this side. Specimens containing no vacuoles showed large vitamin C granules scattered indiscriminately through the cytoplasm. A further specimen showed a number of small granules aligned along one side of the organism as if they were the basal granules of the cilia, but this could not be definitely determined. The other granules in this specimen were large and irregularly placed throughout the cytoplasm.

In the Diatoms examined, a few granules could be seen in the interior and a few were observed in the pores of the valves—again giving the impression of excretion.

Lichen.

A specimen of a crustaceous lichen was treated with the reagent, and "hypo" in turn, washed, dehydrated, and imbedded. Sections of 3μ thickness were cut.

The distribution of the reduced vitamin C is best seen in fig. 25. A few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granules were present in the cells constituting the algal component of the lichen —some appeared to contain no granules, but this was probably due to the fact that they were contained in other sections of the same cell. In the fungal hyphae the granules were seen to be situated in the centre of the hyphal strand and were orientated in a linear fashion.

Algae.

In the specimen of the Cyanophyceae examined—*Oscillatoria*—a rather interesting result was obtained. The granules of vitamin C in the segments of strands examined were in the form of small granules scattered in the central portion of the cell. In the segment of the tip of the strand—presumably the growing tip—the granules of vitamin C were arranged in regular lines radiating back from the tip (fig. 26). The significance of this result is not yet apparent, but it is of considerable interest that Bessey and King (1933) have found a tendency for vitamin C to be present in a higher concentration in the tissues of young, rapidly-growing animals than in those of older animals on an identical diet. It would appear, then, that the vitamin may be associated with protoplasmic synthesis.

Two members of the Chlorophyceae were examined—*Spirogyra* and *Scenidesmus.*

Vitamin C granules were found to be scattered throughout the protoplasmic portion of the cell. Aggregation around the pyrenoids could be observed but no perinuclear aggregation was apparent. A zygospore of *Spirogyra* was also examined and a number of fine granules was found to be scattered throughout the cytoplasm.

The component cells of *Scenidesmus* also exhibited indiscriminate disposition of fine granules with no nuclear relationship.

Fungi.

Various species of *Fusarium* were examined. In the conidia of *Fusarium culmorum* the granules were found to be aggregated at one end, but no specific aggregation could be found in the conidia of *F. martii.* The distribution of the

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Fig. 24. The distribution of the granules in a diatom. **Fig. 25.** The disposition of the granules in a specimen of lichen: A, Algal element of lichen; B, Fungal hyphae. **Fig. 26.** Showing arrangement of granules in ordinary cells and in apical cell of *Oscillatoria.* The arrangement of radial lines in the terminal cell may be easily seen. [× 2,000.] **Fig. 27.** *Spirogyra.* An aggregation of granules may be seen around the pyrenoids: A, Chromatophore; B, Lining of protoplasm round cell; C, Pyrenoid; D, Nucleus; E, Supporting protoplasmic strands for nucleus. **Fig. 28.** Zygospore of *Spirogyra*: A, Nucleus; B, Chloroplasts. **Fig. 29.** Scenidesmus: A, Individuals; N, Nucleus of Individual. [× 5,000]). **Fig. 30.** Conidium of *Fusarium culmorum.* **Figs. 31 and 32.** New growing hyphae of *Helminthosporum sativum.* Granules appear aggregated towards growing end. **Fig. 33.** Old hypha of *Helminthosporum sativum.* Showing only a few granules. **Fig. 34.** *Fusarium martii.* Conidium. **Figs. 35 and 36.** *Ophiobolus graminis.* Specimens of hyphae. **Fig. 37.** *Coletrotrichum phoniodes.* Old hypha, showing a few granules. **Fig. 38.** *Fusarium scirpii.* Hypha. **Figs. 39 and 45.** *Trichoderma lignorum.* Conidia. **Fig. 40.** Epithelial cell from skin of tadpole in culture. **Figs. 41 and 41.** Liver cells of tadpole in culture. **Figs. 43, 44, and 46–49.** Showing distribution of granules in yeast cells.

granules in *Fusarium scirpii* may be seen from the figures, and those showing the distribution of vitamin C granules in *Ophiobolus graminis, Coletrotrichum phonioides, Trichoderma lighorum,* and *Rhizoctonis solania* are also self-explanatory.

Figs. 50, 51, and 52. Sections of *Chlamydomonas* showing disposition of granules in various specimens. [× 1,000.]

The disposition of the vitamin C granules in the young growing portions of the hyphae of *Helminthosporum sativum* is of particular interest. Once again the granules appear to be concentrated towards the growing tip, very few granules being left in the old part of the hyphae.

Specimens of yeast were treated with the reagent and examined whole, without sectioning. The number and size of the vitamin C granules were again very variable, some cells containing only a few large granules and others a large number of small granules. One cell was observed which possessed only one large granule attached to the periphery of the nucleus. Many small vacuoles were also observed in some of the cells, but the small granules of the vitamin showed no special association with them. One cell exhibited an aggregation of the vitamin C granules around one pole of the nucleus.

The mycelia of *Mucor* and *Penicillium* also showed the presence of a number of black granules after treatment with the reagent.

In some of the cultures in which the Protozoa were grown, a very large number of bacteria, chiefly *Bacillus coli communis*, were observed. Smears of these bacteria after treatment with the reagent showed in most cases a single black granule situated at one end of the rod. In some of the organisms a black granule was seen at either end, giving the impression of a *Bacillus diphtheriae*.

Culture of Tadpole Tissue.

Pieces of liver and skin from a tadpole were explanted into a medium composed of normal saline containing small quantities of malted milk powder and marmite. Successful migration of the cells was obtained using this medium.

In all the cells, large well-formed granules of argentophil material were observed after some minutes' treatment with the reagent. They showed no particular orientation in the cell and varied in number. Granules were also observed in a number of migrating connective tissue cells which were found in the cultures. In these cells also the granules appeared to be fortuitously disposed in the cytoplasm. No intra-nuclear granules could be seen in any of the cells of the connective tissue, liver, or in the epithelial cells of the skin.

DISCUSSION.

In assuming that the granules of reduced silver seen in the cells of these lower forms of life represent the form and position of reduced vitamin C, the risk that they may represent some other reducing substance is taken. It must be remembered, however, that there is no naturally occurring substance known apart from ascorbic acid which is capable of reducing the acetic acid-silver nitrate solution in the dark at room temperature. Then also the appearance of the granules—their shape, and their disposition through the cell—are very similar in these lower forms to the granules recognized as the site of vitamin C in the cells of the higher Mammals. If these granules do not represent vitamin C they represent an equally potent, but completely unknown reducing substance. All the evidence at our disposal is in favour of the fact that these granules in the lower organisms, like those of the higher animals, represent the form and position of reduced vitamin C in the cells.

It appears, then, that vitamin C is universally distributed throughout the plant and animal kingdoms. Every cell of the body of an animal has been shown to possess some vitamin C, and it has been shown to be present in higher plants by numerous workers. In this paper it is believed that it has been shown to occur in the Protists (unicellular animals and unicellular plants), in algae and in fungi and in lichens; even certain bacteria have been found to contain a reducing substance with staining properties like those of reduced vitamin C.

The conclusion derived from these results is, therefore, that vitamin C may be necessary for the existence of living protoplasm. Its chemical nature, which enables it to take part in oxidation-reduction systems, would be of inestimable value in expediting chemical reactions in tissues. By a number of authors it is believed to function in cell respiration. Bessey and King (1933) state that a study of the distribution of vitamin C in the tissues of different types of animals provides striking evidence of "its close relation to the normal respiratory processes. In addition (or what is perhaps a corollary) to the relationship between respiratory activity and vitamin concentration there is an evident close relationship between the latter and the presence of complex lipids". From histological and cytological evidence one of us (1933 and 1934) has suggested that vitamin C plays a part in the synthesis of the hormones of the corpus luteum, the adrenal and pituitary glands, and the interstitial cells of the ovary and testis. Vitamin C is believed to form a "redox" system with glutathione, and this system is itself believed to be catalyzed by iron and copper and a thermolabile enzyme. In cells such as the adrenal or corpus luteum the additional complication of unsaturated and saturated fatty acids, fats and lipoids, serves to make an understanding of the problem even more difficult.

Bessey and King (1933) showed that in plants the distribution of vitamin C closely paralleled the distribution of chlorophyll.

Giroud, Rakoto, and Leblond (1935) have even found the chloroplasts of higher plants to stain with the silver nitrate reagent, although this has not been observed by the present author in *Chlamydomonas* or in *Spirogyra*. Giroud et als. confirmed Bessey and King in their work on the parallel distribution of vitamin C and chlorophyll. They found also that plants such as mushrooms contained only very small quantities of the vitamin. These authors feel convinced that the presence of vitamin C in quantity in green leaves and its frequent association with carotinoid pigments, make it almost certain that it plays an important part in photosynthesis, acting (according to Bessey and King) "as one of the many oxidation-reduction factors correlated with the function of chlorophyll". Should vitamin C be essential for the functioning of chlorophyll its importance to the higher plants may be easily realized. But it is not proved that vitamin C is essential for photosynthesis, and Sah (1933) brings forward the obvious counter hypothesis that vitamin C is formed as a result of the photosynthetic action of the chlorophyll. He suggests that the vitamin has the same origin as the sugars, to

which it is structurally related, and he derives them all from activated formaldehyde, a product of the photosynthetic action of the chlorophyll. Vitamin C may certainly be synthesized by living tissues from certain sugars, and mannose appears to be the best suited for this purpose. Guha and Ghosh (1934 and 1935) have found rat tissues able to synthesize vitamin C from mannose both in vitro and in vivo, and Ray (1934) has obtained similar results for seedlings. Should vitamin C be related to the correct functioning of photosynthesis this role would simply be a specialized adaptation of its oxidation-reduction ability.

Even the lowliest organisms contain some granules of argentophilic material, and since, to date, no protoplasm has been found without some granules of this substance it may be permissible to assume that this reducing substance so universally distributed throughout the whole of the plant and animal kingdoms is of fundamental necessity for the continuance of living protoplasm.

The relationship of vitamin C to the actively-growing regions in plants and animals is of considerable interest. Virtanen, von Hausen, and Saastamoinen (1933) have found that the vitamin C content of plants increases rapidly with growth until the time of flowering. Bessey and King (1933) have found that vitamin C shows a higher concentration in young growing animal tissues than in older tissues, and the present author has shown how the vitamin C tends to concentrate in the young growing tips of some fungal hyphae, while only a few granules remain in the older portions. The curious alignment of the vitamin C granules in the growing tip of *Oscillatoria* is also a point of great interest.

SUMMARY.

Vitamin C or an equally potent but unknown reducing substance is shown to be present in Protista, Algae, and Fungi. In view of these results and those recorded in previous papers it is suggested that vitamin C is essential for the existence of living protoplasm.

The tendency towards the concentration of granules of the vitamin around the pyrenoids in *Spirogyra* supports the view of previous authors that it is associated with the photosynthetic activity of chlorophyll.

The aggregation of the granules of vitamin C in the growing portions of these lower forms is in accord with the findings of other workers.

No excretion of oxidized or reduced vitamin C into the culture medium was observed in the case of *Chlamydomonas*, various ciliates, or *Bacillus coli communis*.

ACKNOWLEDGMENT.

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PRELIMINARY OBSERVATIONS ON THE CHEMISTRY AND PHARMACOLOGY OF THE ALKALOIDS OF *DUBOISIA HOPWOODII*

by

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In the 60's of last century the exploration party of Burke and Wills brought back from the interior of the Australian Continent a new plant which was classified by von Mueller (1860) as *Anthocercis Hopwoodii* among the Solanaceae. Bancroft (1872) obtained some specimens of "pituri"—a name given by the Australian aboriginals in those regions to certain leaves used for chewing on account of their narcotic effect; and it was identified by von Mueller and Bailey in 1876 as being leaves of *Anthocercis Hopwoodii*. The identification of a capsular fruit of this plant, and the discovery of a poisonous principle in the leaves, led von Mueller to transfer the plant to the genus Duboisia, family Solanaceae, under the name of *Duboisia Hopwoodii*. This is identical with *Duboisia Pituri* (Bancroft). In 1872 Bancroft obtained sufficient pituri to enable him to prepare an aqueous extract which on being injected into animals (dog, cat, and frog) produced the following results: (1) Preliminary excitement, and rapid respiration, with salivation and vomiting in cats and dogs, and retraction of the eyeball in dogs; (2) Irregular muscular action leading to convulsions; (3) Either death from respiratory paralysis or recovery beginning with slow sighing respirations.

From another specimen received in 1877 Bancroft prepared a brown oil dissolving in water and having the pharmacological effects already described. He was able to show that unlike the extract from *Duboisia Myoporoides*, it produced no mydriasis, although retraction of the eyeball was noted in dogs. Von Mueller (1879) confirmed the existence of this oil, and established its identity as an alkaloid, by the usual precipitation tests with gold and platinum chlorides, picric acid, sodium phosphomolybdate, etc., concluding that: "Piturine is in some respects allied to nicotine, but more closely akin to the duboisine of *Duboisia Myoporoides* (R.Br.), the latter being of lighter colour, of bitter, not acrid taste, of fainter

[1] Hoffmann la Roche Scholar.

odour, less irritating to the eyes and respiratory passages. Its hydrochloride in solution is not precipitated by chloride of platinum, but is so by phosphowolframate of soda, and the precipitate is not re-dissolved by superabundance of that reagent". (Duboisine, the alkaloid of *Duboisia Myoporoides*, was shown by Merck (1892) to contain l-scopolamine, l-hyoscyamine and pseudohyoscyamine (norhyoscyamine). His failure to secure the platinichloride was doubtless due to the presence of excess of the alkaloid).

Ringer and Murrell (1878) received some *unidentified* "pituri" and subjected a 5 p.c. aqueous extract prepared therefrom by Gerrard (1878) to physiological tests. Their statement, in view of the subsequent confusion that has surrounded this subject, is worth giving verbatim: "As we had already shown that *Duboisia Myoporoides* acts in most respects like Atropia, the fact of 'Pituri' being a solanaceous plant, and moreover a Duboisia, led us to expect that 'Pituri' would manifest the properties of Atropia, and our experiments confirm this conclusion. We find that 'Pituri' causes drowsiness, *dilates the pupil* (the italics are ours), dries the mouth, produces general weakness with convulsive twitchings, and antagonizes the action of muscarine on the heart. It possesses, however, two properties distinct from Atropia; it produces sickness and increases salivary secretion, in large doses, before it dries the mouth." In a second paper (1879) they state that "piturine" is more closely allied to nicotine than to the solanaceous alkaloids. Fraser (1879) described the results of physiological tests, from which he concluded that, although similar to nicotine, the alkaloid was, in fact, not nicotine, largely owing to difference in its action upon the eye. Petit (1879) obtained from Bancroft some 50 gm. of "pituri", from a study of aqueous extracts of which he concluded that the alkaloid present was nicotine. He based his conclusion on the following results:

(1) Optical Rotation (0·236 gm. in 10 c.c. 98 p.c. ethyl alcohol; 20 cm. column, sodium light) = — 123·9° Nicotine = — 121·9 (Buignet).

(2) Alkalimetric strength.

(3) Chloroplatinate (flattened prisms with parallelogram base)

Pituri:	Platinum = 34·0 p.c.	Chlorine 36·0 p.c.
Nicotine	„ = 34·4 p.c.	„ 37·0 p.c.
(Calculated) for $C_{10}H_{14}N_2 2HCl_2PtCl_2$		

(4) Iodo-nicotine test.

This may be taken as the starting point of the subsequent confusion concerning the identity of the alkaloids of *Duboisia Hopwoodii*. Liversidge (1880) obtained from the Barcoo Creek district a sample of pituri, which without further identification be concluded to be leaves and twigs of *Duboisia Hopwoodii*. He extracted these with boiling water, and distilled after making alkaline with caustic soda, obtaining a substance boiling at 243-244°C. under atmospheric pressure. The freshly-prepared substance was clear and colourless with a nicotine-like smell,

but on standing it became yellow and finally brown, with an odour similar to pyridine. Liversidge's product was volatile, and fumed with hydrochloric acid vapour. The taste was acrid, pungent, and persistent. He could prepare only the oxalate and this not pure, whilst acetate, sulphate, and hydrochloride could not be crystallized. With picric, phosphotungstic, and phosphomolybdic acids and with iodine, he obtained reactions similar to those with nicotine, but unlike nicotine the iodine compound treated with strong caustic soda gave an odour of iodoform. The iodo-nicotine compound melted at 100°C., that of the Duboisia alkaloid 110°C. According to Liversidge the following are the differences between his alkaloid and nicotine:

(1) The platinichlorides and gold chlorides (difference not stated).

(2) Iodoform odour when the iodo compound is decomposed by sodium hydrate.

(3) Palm's Test. Pituri alkaloid does not turn violet when gently warmed with strong hydrochloric acid (Sp. Gr. 1·12) nor turn orange on adding nitric acid.

His combustion results (8 analyses) were: C. 76·58, H. 8·48, N. 14·94, giving relative proportions C. 5·98, H. 7·96, N. 1·00, i.e. 6: 8: 1.

These may be expressed thus:

Calculated.		Found.
$C_6 = 72 = 76·59$	per cent.	76·56 per cent.
$H_8 = 8 = 8·51$	„ „	8·48 „ „
$N = 14 = 14·90$	„ „	14·94 „ „
100·00	„ „	99·98 „ „

An attempt to verify these results by reference to the analysis of the platinum salts proved difficult owing to variations in the composition of these. From 23 analyses he found variations from 34·15 p.c. to 38·40 p.c. of platinum. Seven analyses gave 35·35 p.c.-35·55 p.c. Chlorine in nine analyses 31·32 p.c.-36·86 p.c. From this he concluded that either the salts were not uniform in composition, or that they were unstable. The comparative basicity of nicotine and "piturine" was found to be nicotine = 0·2997, piturine = 0·2986. Langley and Dickinson (1890) compared the physiological actions of nicotine and pituri alkaloid provided by Liversidge, and concluded that they were identical.

Rothera (1910) was asked to investigate a sample of lanceolate leaves and twigs alleged to be a cure for neuralgia when smoked. Finding the physiological effect to be similar to that of nicotine, and being led through this to the subject of "pituri", he had the leaves identified by Professor Ewart, by actual comparison with leaves of a *Duboisia Hopwoodii* growing in the Melbourne Herbarium. This is the first instance of identification being established prior to chemical investigation since the work of von Mueller. He concluded that the alkaloid was nicotine for the following reasons:

(1) Identical physiological reactions.

(2) Volatility.

(3) Positive iodo-nicotine test.

(4) Solubility in water, and particularly loss of opalescence on cooling a warm solution.
(5) Difficulty in preparing crystalline salts: Sulphate and Hydrochloride.
(6) Melting point of oxalate 77°C.
(7) Platinichlorides: (a) Orange, similar to that obtained from nicotine tartrate (Merck); (b) Yellow, obtained after separating (a).

His analyses of these compare with the formulae given for such platinum salts in Beilstein's Handbook, as follows:

(a) $C_{10}H_{14}N_2$, 4HCl, $PtCl_2$:	Platinum 33·91 p.c.	Chlorine 37·18 p.c.
Found for (a) (3 analyses)	„ 34·16 p.c.	„ 32·51 p.c.
(b) $C_{10}H_{14}N_2$, 2HCl, $PtCl_2$	„ 38·8 p.c.	„ 28·4 p.c.
Found for (b) (2 analyses)	„ 35·96 p.c.	„ 32·15 p.c.

Refractive index 15°C. 1·5283. Nicotine (Llandolt): 1·52828.
Optical Rotation 23°C. Pituri = 4·77. Nicotine = 4·60.

COMBUSTION ANALYSIS.

	Pituri.	Nicotine $C_{10}H_{14}N_2$.	Calculated from Liversidge $C_{12}H_{16}N_2$.
Carbon	71·90, 74·23, 73·14	74·06	76·59
Hydrogen	8·56, 8·60, 9·02	8·64	8·51
Nitrogen	16·20, 17·7, 17·5	17·30	14·90

This evidence is not itself sufficient to warrant the conclusion that nicotine is the alkaloid present in the leaves of *Duboisia Hopwoodii*.

HISTORY OF THE PRESENT INVESTIGATIONS.

It will be seen from the foregoing survey of the literature that the "pituri" chewed by the Australian aboriginal has been throughout believed to be the leaves of *Duboisia Hopwoodii*. During the course of anthropological expeditions to Central Australia, it became obvious to Hicks that the real identity of "pituri" was by no means a settled question. It was found by members of the expedition that the natives in the area north, north-west, and south-west of Alice Springs within a radius of 300 miles, chewed, under the name of "pituri", the leaves of at least two varieties of Nicotiana—a large-leafed, most-favoured variety termed *Nicotiana Excelsior*; and a smaller-leafed, stunted variety, *Nicotiana Gossei*. The identity of these has been confirmed by Mr. J. M. Black. It was, moreover, established beyond doubt, that the Australian tobacco variety—*Nicotiana Suaveolens*—is never used, by these natives at least, as the leaves are sticky and the taste is rank c.f. Cloetta (1923). At Mt. Liebig it was found that *Duboisia Hopwoodii* was used to catch emus, by poisoning waterholes at which they drank with a handful of the leaves; yet there was evidence that Duboisia leaves were in some cases mixed with Nicotiana leaves for chewing (evidence gained from Hermannsburg Mission). It was not, however, until 1932 at the Musgrave Ranges, that unequivocal evidence of the use of Duboisia leaves as "pituri" was obtained. A large quantity of the leaves collected for investigation was being dried in the

sun, when the natives gathered round making signs and mentioning the word "pituri". An interpreter being called, Hicks found that they wished to indicate that it was "pituri", but only used when *real* "pituri", i.e. Nicotiana, was unobtainable. At last was disclosed the essential nature of the confusion as to the plant actually used for chewing. The so-called Emununga, or Emu poison *(Duboisia Hopwoodii)* is actually used, albeit in smaller quantities, or admixed with tobacco for chewing by the natives. It is used also as an emu poison, and from the small amount used it is obvious that it is very toxic. Camels, it is well-known, succumb if they eat only one mouthful of the bush torn off during a journey. For an extended review of the historical literature on this subject, see Johnston and Cleland (1934), from whom we differ in stating, on the evidence above quoted, that *Duboisia Hopwoodii* is used as pituri in the Musgrave Ranges area. The "pituri" is chewed at intervals for days, the bolus being passed from one native to another. Each native, before chewing, rolls it in some freshly-prepared wood ash, made by holding a burning mulga (Acacia) twig over a flat stone to catch the ashes. This serves the same hydrolytic purpose as the lime chewed with the Areca nut by the natives of India, etc.

The specimens used in extraction of the alkaloid in the course of this investigation have all been identified by Mr. J. M. Black, and have been collected at Mt. Liebig, Harper's Springs, Kimba, Eyre's Peninsula,[2] and near Ernabella in the Musgrave Ranges.

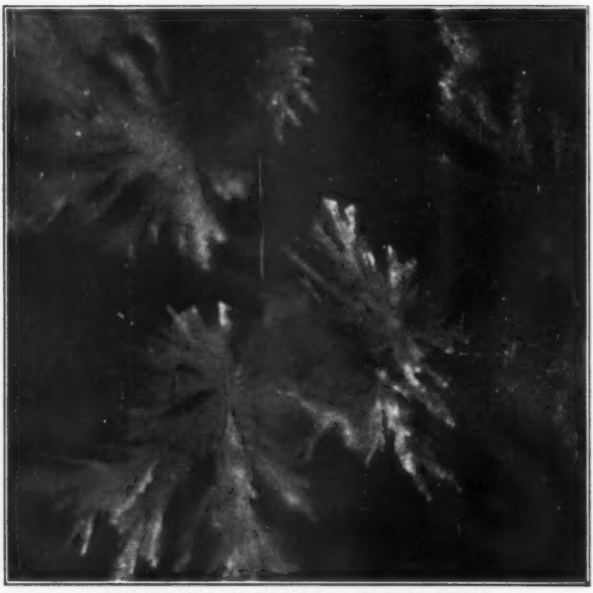
EXTRACTION OF ALKALOID FROM *DUBOISIA HOPWOODII*.

Thirteen kg. of dry, powdered leaf were extracted in a large scale Soxhlet apparatus, with 90 p.c. alcohol, until no further chlorophyll could be removed. The volume was reduced to one-third by concentration in vacuo, and water was added to precipitate chlorophyll, which was filtered off. The reddish-brown filtrate was treated with lead acetate, filtered, and after being made alkaline with sodium hydroxide, was shaken for 6 hours with changes of ether, from which the alkaloid was removed by shaking with weak hydrochloric acid. On making this latter again alkaline, and re-extracting with ether, an extract of the alkaloid was obtained which, after removal of ether and reduction of bulk by distillation in vacuo, gave a residual liquid of 150 c.c. volume. This was fractionated in vacuo in a Perkin triangle apparatus after displacing air by nitrogen, and the receiver was immersed in liquid air. Every precaution was taken to reduce the possibility of decomposition or racemization of any alkaloid present. Several fractions were set aside, but the largest and most clearly-defined one, boiled at 117°C. (Bath Temp. 130°C.) 4·35 mm. It was decided to proceed at once to a further examination of this fraction.

Properties. It is a water-clear alkaline oily liquid (gradually turning a golden to brown colour on exposure to air and light, with a pungent odour recalling that of impure nicotine, but definitely sweeter and distinct therefrom. It dissolves in water, giving an opalescent solution, probably due to impurity which may be grease from the tap of the Perkin's triangle. It is readily soluble in alcohol and ether. It boils at 266-268°C. under atmospheric pressure. Attempts to pre-

[2] We are indebted to Mr. J. E. Pick, of Koondambo Station, for this specimen.

Nicotine Picrate Crystals × 80, M.P. 218°C.



Unknown Picrate Crystals × 80, M.P. 188°C.

pare the hydrochloride in crystalline form were not successful. The most readily-prepared compound is the picrate, which separates immediately, especially in the presence of excess acid. The picrolonate can be prepared from alcoholic solution, and separates only on evaporating some of the alcohol. Aqueous alcohol is most suitable for re-crystallizing the picrolonate. If, to an aqueous solution of the alkaloid, a little 5 p.c. hydrochloric acid is added, and then a solution of platinic chloride, a yellow precipitate forms which on stirring crystallizes to form the orange platinichloride, probably 2 HCl, $PtCl_4$. On adding further platinic chloride until no more precipitate is formed, these crystals can be filtered off, leaving a mother liquor which either on standing or on adding large excess of platinum salt, throws down a further precipitate, which crystallizes on stirring in a yellow form, probably 2 HCl, $PtCl_2$. The aurichlorides were very unstable. The addition of the following substances gave amorphous precipitates as follows: Phosphomolybdic acid (yellow); phosphotungstic (white); silicotungstic (white), The addition of iodine in ether followed by strong caustic soda led, on shaking, to the production of the iodoform odour noted by Liversidge. Nicotine does not give this result.

Refractive Index. This was the first physical constant determined, and the following table shows the values obtained (average readings) on separate fractions obtained at different dates:

First Series (impure).		Second Series (const.B.P.).	
1.	1·5476 19°C.	5.	1·5490 18·6°C.
2.	1·5541 19°C.	6.	1·5518 18·6°C.
3.	1·5509 19·5°C.	Redistilled Nicotine (Merck).	
4.	1·5482 19°C. (purer sample)		1·5294 16·6°C.

It is clear that there is a constantly higher refractive index than that of nicotine in all cases, and the evidence against the correctness of Rothera's conclusions therefore warranted further investigation.

Optical Rotation:

Corrected averages for a series of eight readings on three specimens = 20·60/16°C.; 20·60/17·5°C.; 20·61/16°C.

$$[\alpha]_D^{16^\circ - 17.5^\circ C.} = \frac{20 \cdot 60 \times 10}{1 \cdot 0757 \times 4 \cdot 961} = +38 \cdot 59$$

Nicotine under the same conditions gave $[\alpha]_D^{16^\circ} = -166^\circ$.

(A special micro-rotation tube length = 1·9531 inches was constructed for the purpose.)

Molecular Weight Determined by Rast's Method.

It was found necessary to take at least twelve times as much camphor as alkaloid to secure constant results. The following are the most concordant values:

No.	Wt. of Unknown.	Wt. Camphor.	Depression of M.P.	Mol. Wt. Calculated.
1.	0·002109	0·025491	21·8	151·8
2.	0·001378	0·094390	4·2	139·2
3.	0·000474	0·009035	14·0	149·9
4.	0·000300	0·011326	7·0	150·9
5.	0·003000	0·027031	28·1	148·2
6.	0·001112	0·022462	13·2	149·4
7.	0·001850	0·160211	3·1	147·7
8.	0·001492	0·077584	5·16	149·1

Average = 147·9.
Average omitting No. 2 = 149·5.
Molecular Weight of Nicotine = 162.

Molecular Weight Determined from Analysis of the Platinichloride.

Eighteen estimations were selected out of 38. The combining weights varied between 191·0 and 211·5 in the selected series. The wide variation found confirms the work of Liversidge, who was unable to determine the molecular weight in this manner. It is probable that the inconstant results are due to two types of platinichloride being present, although as far as could be determined, we were dealing with the orange salt with a probable formula of B. 2HCl, $PtCl_4$ with a molecular weight of 130·1, using the average platinum values from the selected series. If the yellow salt, B, 2HCl, $PtCl_2$ were taken, the molecular weight would be 201·1.

Molecular Weight Determined from Analysis of the Silicotungstate.

Calculated from the formula $2B_2 2H_2O$, SiO_2, $12WO_3$ the ash being taken as SiO_2, $12WO_3$. Molecular Weight 2496·0.

No.	Mol. Wt.	
1	151·0	
2	144·5	
3	151·5	
4	147·4	
5	150·1	
6	150·0	Average = 148·9.

Determination of Carbon and Hydrogen.

Alkaloid. B.P. 117°C. at 4·35 mm. pressure.

No. of Analysis.	Carbon per cent.	Hydrogen per cent.
1	71·94	8·34
2	71·71	8·30
3	72·61	8·15
4	72·00	7·99
	Average = 72·06	8·19

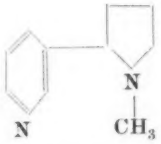
Picrolonate. Recrystallized to give a constant melting point. M.P. = 252-253°C.

No. of Analysis.	Carbon per cent.	Hydrogen per cent.
1	51·78	4·63
2	51·81	4·67
3	51·50	4·33
4	51·55	4·40
	Average = 51·66	4·50

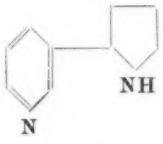
Table showing comparison between the analyses of Nicotine, Nor-Nicotine, and Duboisia Alkaloid.

Compound.	Nicotine $C_{10}H_{14}N_2$.	Nor-Nicotine $C_9H_{12}N_2$.	Unknown.
Alkaloid	C = 74·08 p.c.	C = 72·97 p.c.	C = 72·06 p.c.
	H = 8·64 p.c.	H = 8·11 p.c.	H = 8·19 p.c.
Picrolonate	C = 52·17 p.c.	C = 51·47 p.c.	C = 51·66 p.c.
Diprierolonate	H = 4·35 p.c.	H = 4·14 p.c.	H = 4·50 p.c.

It is evident that the results point to a nicotine-like molecule of smaller molecular weight, and that the absence of one methyl group would explain the difference found thus:



Nicotine
Mol. wt. 162

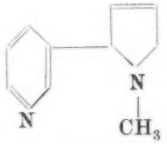


Nor-Nicotine
Mol. wt. 148

In collaboration with Professor Ernst Späth, Wien, the alkaloid was oxidized with potassium permanganate to yield a crystalline product, which on recrystallization, gave when mixed with pure nicotinic acid no lowering of the melting point of that substance. The molecule is therefore a β-substituted pyridine derivative, and in all probability is *d*-nor-nicotine. The absorption spectrum in the ultra-violet region is what would be expected were this the case. The absorption coefficient is slightly lower, but the maximum absorption is at the same frequency as that for nicotine. Maximum = 2,600. Nicotine: Maximum = 2,604.

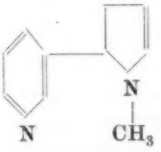
The natural occurrence of a possible *d*-nor-nicotine in one of the Solanaceae, not only confirms the early suspicion of von Mueller and of Liversidge, but also completes an interesting phase of modern investigation in the nicotine group of alkaloids. Pictet (1901) described among other alkaloids present in tobacco, on rather insufficient grounds, a substance to which he gave the name nicoteine. It was believed to be a pure compound, boiling at 266-267°. Its characteristics were given as $[\alpha]_D^{17^\circ} = -46{\cdot}41$. $D_{4^\circ}^{12{\cdot}5} = 1{\cdot}0778$. $n_D^{14^\circ} = 1{\cdot}5602$. M.R. = 48·01. By oxidation it yielded nicotinic acid, i.e. it contains a β-substituted pyridine ring. Its instantaneous decolourization of $KMnO_4$ in H_2SO_4 solution, led him to ascribe to the substituted portion a methylated and partially-reduced pyrol ring. The presence of an assymetric carbon atom left only two choices of formulae:

I



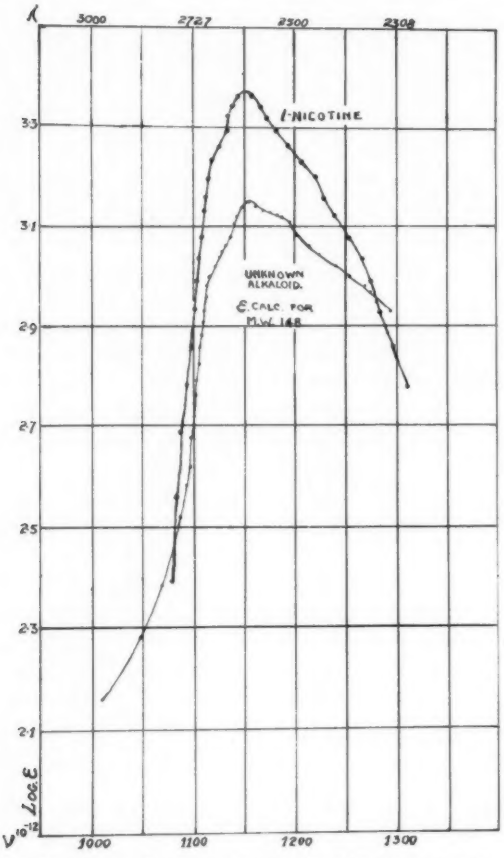
Dihydro Nicotyrin
(Pictet)

II



Nicoteine
(Pictet)

and since I had already been accepted by him for dihydronicotyrin, only II remained.



Graph 1. Ultra-Violet Absorption Spectrum Curves for *l*-Nicotine and the Duboisia Alkaloid.

Ehrenstein (1931) recently investigated this fraction of the tobacco alkaloids by fractionating under reduced pressure, and found a substance with constant boiling point at 269-270°C. at ordinary pressure. Further investigation pointed to the existence in this of an alkaloid that appears to be *l*-nor-nicotine, which Braun (1930) believed he obtained by a synthetic method. Polonovski's (1927) synthesis is disputed by Braun and by Ehrenstein.[3] A tabular comparison of these compounds with that isolated from the leaves of *Duboisia Hopwoodii* is instructive and points to the existence of dextro and laevo-nor-nicotine, and a steric analogy with not only nicotine, but also the natural amino acids c.f. Karrer (1926).

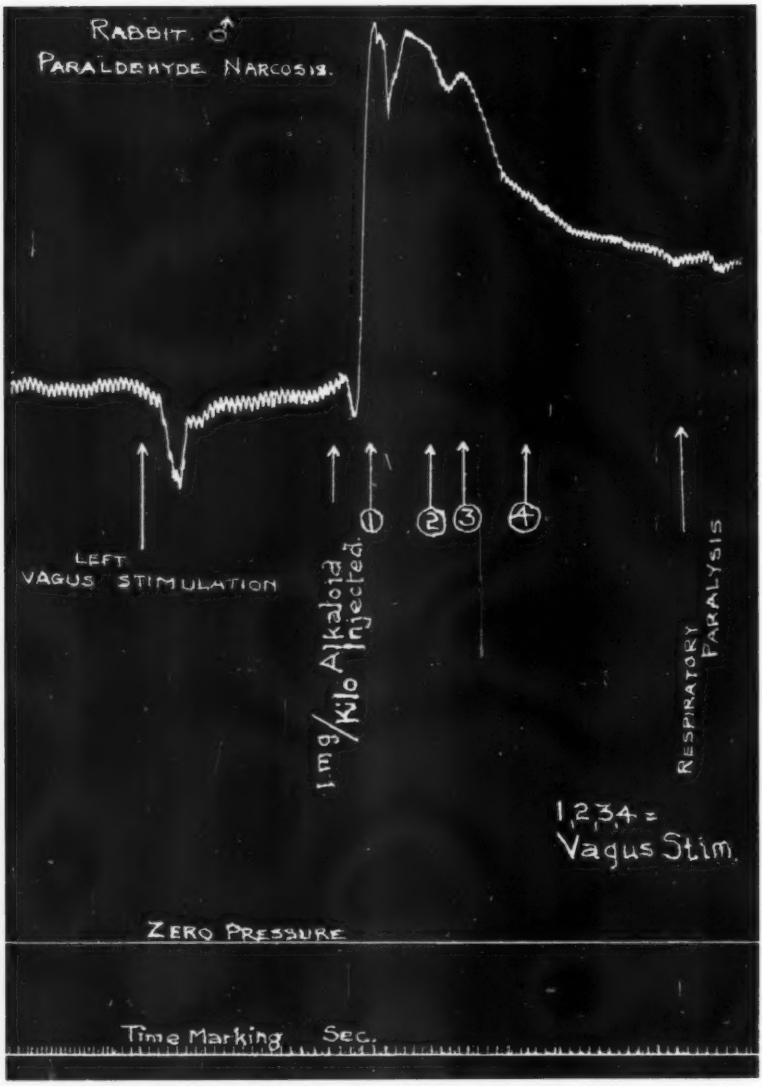
[3] Späth, in a private communication, also throws doubts on this synthesis.

Table

Property.	Nicotine.	*l*-Nor-Nicotine (Ehrenstein).	*r*-Nor-Nicotine (Braun)	Duboisia Alkaloid.
Boiling Point	247° 760 mm.	266–267° 760 mm.	139·0–140° 12 mm.	117° 4·3 mm. 150° 33 mm.
	*124° 23 mm.	130·5–131·3° 11 mm.	266–267° 760 mm.	266–268° 760 mm.
Density	$*D_4^{10} = 1·0174$	$D_4^{19·5} = 1·0737$	$D_4^{19·5} = 1·044$	$D_4^{10} = 1·0757$
	$D_4^{10} = 1·0097$			
Refractive Index	$n_D^{15} = 1·5283$ (Llandolt)	$n_D^{18·5} = 1·5387$		$n_D^{18·3} = 1·5490$
	$*n_D^{16} = 1·5294$			
Optical Rotation	$[\alpha]_D^{19} = -166$	$[\alpha]_D^{20} = 17·7$	$[\alpha]_D^{20} = -55$	†$[\alpha]_D^{16} = +38·59$
Picrate	M.P. 218°	M.P. 191–192°	M.P. 188–190°	M.P. 186–188°
Picrolonate	M.P. 213°	M.P. 250–252°	M.P. 223–225°	M.P. 252–253°
Mol. Wt.	162	148	148 (?)	148
Ultimate Analysis Alkaloid	C = 74·08 H = 8·64	C = 72·94 H = 8·11	—	C = 72·06 H = 8·19
Picrolonate	C = 52·17 H = 4·35	C = 51·47 H = 4·14	—	C = 51·66 H = 4·50
Absorption Maximum	$\lambda = 2604$ log. ϵ = 3·37	—	—	$\lambda = 2600$ log. ϵ = 3·15
(M.L.D.) Rat I.P.	0·0045 gm.	—	—	0·0017 gm.

* Re-determined Values.

† Probably mixed with racemic nor-nicotine, since Späth has now isolated *l*-nor-nicotine from tobacco with a specific rotation of $[\alpha]_D = -88·8°$. (Private communication, 22-7-35.)



Blood pressure curve. Rabbit. Showing effect of injection of Duboisia poison (*d*-nor-nicotine). It will be noted that following peripheral stimulation of the splanchnic ganglia, resulting in secretion of adrenaline, the vagi become paralyzed within 70 seconds; respiration being paralyzed about 15 seconds later. Failure of blood pressure to return to normal is due to asphyxia.

It would appear that Ehrenstein's compound is rather racemized, whilst that of Braun is almost completely so.

Toxicology:

The minimum lethal dose was estimated by peritoneal injection into rats, using series of 32 animals and comparing with nicotine used in the same manner. It was found that the quantities of nicotine and the unknown which produced the same percentage of deaths were 0·0045 gm. and 0·0017 gm. respectively, i.e.

$$\text{Unknown (M.L.D.)}\ \frac{\text{Rat}}{\text{I.P.}} = 0{\cdot}0017 \text{ grams} \pm 12{\cdot}4\%$$

$$\text{Nicotine (M.L.D.)}\ \frac{\text{Rat}}{\text{I.P.}} = 0{\cdot}0045 \text{ grams} \pm 13{\cdot}4\%$$

Effect on Isolated Perfused Mammalian Heart and on the Blood Pressure.

0·4 c.c. of an M/100 solution added to the perfusing fluid caused a preliminary augmentation of beat of both auricles and ventricles followed by rapid onset of bundle block, leading at its height to an A/V ratio of 16/6. Smaller doses slowed the rate of the whole heart. Recovery from the bundle block was complete. The action is thus similar to that of nicotine. Intravenous injection of 2 mg. into the intact animal (rabbit) caused a rise of blood pressure similar to that due to adrenalin. During the falling phase the vagus became paralyzed. This effect is similar to that produced by the stimulation of the splanchnic ganglia by nicotine.

Action on Isolated Frog's Gastrocnemius Preparation.

The action is similar to that of nicotine, successive maximal stimuli applied to the nerve leading to smaller response until paralysis is complete.

It is, however, evident that there are some quantitative and qualitative differences in the physiological action of the alkaloid. The symptoms of poisoning in the rat were distinct from those produced by nicotine, although in both cases death was due to respiratory failure.

Table of Symptoms Due to M.L.D.

Nicotine.	Unknown.
20 seconds after I.P. injection.	30-60 seconds after I.P. injection.
Breathing slowed.	Breathing slowed.
Onset of tremors of body.	Jerky lifting movements of head.
Back becomes concave. Tail circles over and touches head.	Hind legs become weak, and are dragged after the body.
Animal lies on side. Limbs move convulsively.	Back becomes arched (convex), animal falling on its paralyzed hind quarters. Front limbs normal.
Back now arches and hind legs fold under abdomen. Tail sinks, and legs are thrust out backwards with each respiratory spasm.	Jumping type of convulsion in which only front legs are able to take part.
	Abdominal muscles intensely contracted to give scaphoid abdomen. Back remains arched throughout.

SUMMARY.

1. The chief alkaloid present in the leaves of *Duboisia Hopwoodii* is in all probability *d*-nor-nicotine, and the chemical evidence for this conclusion is given in detail.

2. A brief introduction to its pharmacology is cited, and this supports the chemical evidence.[3]

ACKNOWLEDGMENT.

Our thanks are due to Prof. J. G. Wood for his assistance in the botanical identification of specimens.

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[3] A detailed study of the pharmacology of this substance will appear elsewhere, and in collaboration with Professor E. Späth, Vienna, a further account of the chemical relationships of the alkaloid will be published in the Berichte der Deutschen Chemischen Gesellschaft.

COMPOUNDS OF PROTEINS WITH O-NITROPHENOLS

by

ADOLPH BOLLIGER

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(Submitted for publication 1st June, 1935.)

Recently it has been shown (Bolliger and Goulston, 1935) that globin and haemoglobin form a well-defined and identical globin picrate which can be readily analysed for its picric acid content by titration with methylene blue. In the present communication the preparation of similar compounds of globin with other o-nitrophenols, namely picrolonic acid and 2-4 dinitro *a*-naphthol, are described. Furthermore, two other proteins, edestine and casein, have been treated in the same way with the three o-nitrophenols mentioned.

TECHNIQUE.

Two gram quantities of the proteins were added to 100 c.c. of a hot, approximately 3 p.c. alcoholic solution of the o-nitrophenol. The mixture was then kept on the water bath for one hour, with occasional stirring. Globin was added in the form of haemoglobin, and edestine and casein were used in the form of dry, fine powders supplied by Hoffmann La Roche. In all cases the substance added was converted into a yellow compound which was of a much deeper yellow in the case of the experiments with 2–4 dinitronaphthol. After cooling, the precipitate was filtered off by suction, and was washed first with a small amount of alcohol, and then with ether and benzol. The substance was then dried in vacuo and analysed for its o-nitrophenol content, as described in the case of globin picrate in a previous communication (Bolliger and Goulston, 1935).

The results (with duplicates) obtained by this technique are listed in Table 1.

DISCUSSION.

The o-nitrophenols used in this investigation, namely picric acid, picrolonic acid, and 2-4 dinitronaphthol, were those which on previous occasions had been found to be most suitable for titration with methylene blue (Bolliger, 1934). Globin reacts with an alcoholic picric acid solution at room temperature. Under these conditions, however, the reaction of globin with picrolonic acid, which is a weaker acid, is unsatisfactory and the mixture has to be warmed. A picrolonate of globin is then obtained, which on analysis shows a picrolonic acid content of 25 p.c., which would correspond to a picric acid content of about 22 p.c. This figure is practically a constant one, and even after warming the mixture on the water bath for one or several hours, no material increase in the picrolonic acid content is ob-

tained. Under very prolonged treatment the picric acid content of globin picrate may become slightly higher than mentioned in the table, but no value above 27 p.c. has been obtained, even after warming for eight hours in a picric acid solution containing 4 p.c. picric acid. In the previous communication (Bolliger and Goulston), the picric acid content of globin picrate averaged 25·7 p.c. In the present work, under different experimental conditions, the average content was found to be 26·0 p.c.

One may therefore assume that in compounds such as the picrate and picrolonate of globin the combining power between the protein and the acid approximates a maximum. In the case of other proteins of a more acid nature, such as casein and edestine, such saturated compounds could also be obtained. As with the picrolonate of globin, treatment in the cold or heating for only short periods leads—particularly with casein—to incompletely saturated compounds. Consequently, for purpose of comparison, all the three proteins mentioned were treated alike with the respective nitrophenol. After heating at water-bath temperature for one hour in a markedly excessive amount of a 3 p.c. alcoholic solution of the o-nitrophenol, practically saturated compounds or salts of the three proteins were obtained in every instance. Prolonged treatment, such as heating for 12 hours in a higher concentration of the acid, either did not increase the nitrophenol content materially, or by not more than 0·6 p.c.

These compounds of protein are dissolved by alkalies and are hydrolyzed in the presence of water. They are slightly soluble in organic solvents, but practically no bound nitrophenol is washed away with anhydrous solvents such as ether and benzol. The accuracy of the o-nitrophenol determination is satisfactory. Duplicate determinations differ by a maximum reading of 0·5 p.c. As shown in Table 1, the weaker acids combine to a lesser degree with proteins than the stronger acids. The combining power of picrolonic acid is less than that of picric acid, while 2-4 dinitrophenol, which is still less acid, has a smaller combining power than picrolonic acid. This is in agreement with the work of Ishiyma (1929). On the other hand, proteins show diminished combining power with increasing acidity. Edestine combines to a lesser degree than globin, and casein shows a combining power still lower.

TABLE 1.

Combining power of proteins with o-nitrophenols expressed in p.c. of o-nitrophenol present in the o-nitrophenolate of protein.

	Picrate p.c. Picric Acid.	Picrolonate p.c. Picrolonic Acid.	Dinitronaphtholate p.c. 2–4 Dinitronaphthol.
Globin	26·0	24·7	18·0
	25·9	25·1	17·7
Edestine	24·2	23·4	
	24·6	23·9	
Casein	21·1	19·0	13·2
	20·8	19·0	13·7

Proteins in fresh biological material other than haemoglobin form the corresponding nitrophenol compounds if the water content of the reaction mixture is kept below 10 p.c. The picrates of the proteins in milk, human serum, and egg-white were prepared and analysed for their picric acid content, the respective percentages being 20·5, 22·0, and 23·4.

No comment will be made with regard to the nature of the union between protein and o-nitrophenol, but renewed attention is called to the fact that proteins, even if of an acid nature, such as edestine and casein, form approximately saturated compounds of remarkably constant composition with o-nitrophenols, and in all probability with other acids also.

With β-naphtholine sulphochloride, Edlbacher and Fuchs (1921) obtained protein compounds which were also characterized by their constant composition.

SUMMARY.

Edestine and casein, as well as globin, form compounds with o-nitrophenols such as picric acid, picrolonic acid, and 2-4 dinitro-α-naphthol.

The o-nitrophenol content of these compounds can be readily determined.

Under suitable conditions compounds are obtained which approximate a state of saturation. These "saturated" compounds are of remarkably constant composition.

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PHOTOCHEMICAL OXIDATION OF HAEMOGLOBIN

by

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The production of methaemoglobin from haemoglobin through irradiation with ultra-violet light appears to have received very little attention since this observation was recorded by Hasselbalch (1909). His investigations demonstrated that short wave-length electro-magnetic waves, particularly in that portion of the ultra-violet region between 2,500 Å.U. and 2,000 Å.U., were singularly potent in their destruction of the oxyhaemoglobin contained in defibrinated blood. Among the products of this action were found methaemoglobin and smaller amounts of haematin, denatured globin, and haemochromogen. Since methaemoglobin formation had previously been shown to be effected by ozone, the possibility of conversion of oxygen to ozone was given due consideration, but was rejected on account of the absence of reduction in volume of gas which must accompany this change. Certain dyestuffs, notably eosin, were shown to exert a sensitizing effect on haemoglobin transformation which was most readily detected when both air and light were admitted.

During an investigation on the changes taking place in meat during storage involving the action of light, the production of methaemoglobin by ultra-violet light was studied, and attempts were made to accelerate the reaction by adding small amounts of likely sensitizers. The remarkable effect of one of these, namely potassium iodide, is apparent from the results of the following experiments.

MATERIALS AND METHODS.

The haemoglobin solutions were prepared by separating red blood corpuscles from ox blood by centrifuging and washing, the cells being then laked by the addition of three volumes of distilled water. For the exposure rubber-stoppered silica test tubes, 5 in. x ½ in. were employed, control tests being carried out simultaneously in tubes of ordinary soda glass with blackened exterior surfaces. Later, clear glass was found to be equally effective in blocking the transmission of active rays, and the practice of blackening the outside was discontinued.

Throughout the experimental work salts of A.R. quality only were used.

Specimens were irradiated by holding the tubes at a distance of two feet from a "Hanovia" mercury vapour lamp, and in the path of the direct rays. Unless otherwise indicated, a standard exposure period of four hours may be assumed.

To test for the production of methaemoglobin 1 ml. aliquot portions of the irradiated and

control solutions were diluted to 20 ml., complete oxygenation of any reduced haemoglobin ensured by shaking with air, and the resulting solutions examined with the direct-vision hand spectroscope. The degree of oxidation of haemoglobin was determined by comparison against the control solution of the degree of weakening of the two oxyhaemoglobin bands situated in the green at 5,780 A.U. and 5,400 A.U. respectively, and the intensifying of the characteristic band of methaemoglobin at 6,300 A.U. wavelength in the red portion of the spectrum.

Photographs were taken of the absorption spectra on Kodak pan-chromatic plates, using as a source of illumination a 50 candle-power electric light bulb shrouded in tissue-paper.

IRRADIATION EXPERIMENTS.

Experiment 1. Illumination of oxyhaemoglobin in 0·1 molar potassium iodide solution, with the mercury vapour lamp, resulted in the development of an intense methaemoglobin spectrum, whereas in a control solution containing only oxyhaemoglobin the photo-chemical oxidation was very much slower. By increasing the time of irradiation of the iodide-containing solution from four to nine hours, a slightly more intense absorption due to methaemoglobin was apparent, but at no stage to the same degree as when methaemoglobin was prepared by adding potassium ferricyanide to a fresh portion of the original haemoglobin solution.

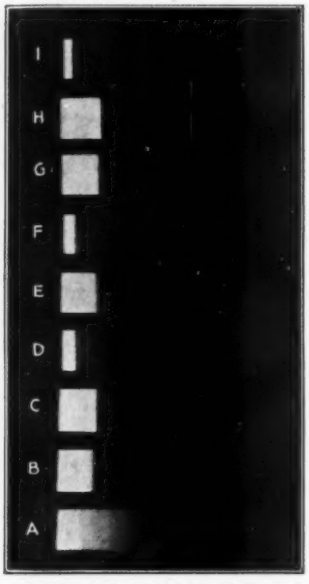
Experiment 2. In the presence of 0·01 molar potassium iodide, irradiation produced a negligible proportion of methaemoglobin. An increase in concentration from 0·1 molar to 0·5 molar produced a much more rapid oxidation than occurred in 0·1 molar potassium iodide solution, but was accompanied by considerable protein precipitation. The resultant opacity was removed by centrifugation before comparison with the control solution. On account of the complication arising from the use of potassium iodide in concentrations exceeding 0·1 molar, this proportion was employed in all subsequent tests.

Experiment 3. Following the addition of a dipotassium hydrogen phosphate and citric acid buffer mixture of the same pH value as blood, i.e. at pH 7·4, no diminution in the extent of methaemoglobin formation was apparent, on exposure, in comparison with unbuffered control samples.

Experiment 4. Irradiation of an aqueous 0·5 molar potassium iodide solution under standard conditions was accompanied by a slight liberation of iodine.

Experiment 5. By laking the corpuscles with a saturated aqueous iodine solution and irradiating in the absence of potassium iodide, no greater conversion to methaemoglobin was detectable than in solutions containing no iodine. A similar oxyhaemoglobin-iodine preparation containing 0·1 molar potassium iodide, and another in which the free iodine concentration had been increased to 0·05 molar, were both found to exhibit changes identical with those which characterize solutions containing no iodine. Iodine concentrations in excess of 0·05 molar could not be conveniently tested, for the solutions were rendered almost opaque, and the relative intensities of spectrum absorption were therefore impossible to distinguish.

Experiment 6. By deoxygenating an oxyhaemoglobin solution containing 0·1 molar potassium iodide with a vacuum pump, and then sealing the tubes under reducing conditions, a slightly more extensive conversion to methaemoglobin was achieved on subsequent irradiation than occurred in a sample containing the same concentrations of potassium iodide and blood pigment in the completely oxygenated condition. In the absence of potassium iodide, a control solution containing reduced haemoglobin elicited no detectable change on exposure.



I. Comparison solution containing pure methaemoglobin, prepared artificially by the addition of potassium ferricyanide to a portion of the oxyhaemoglobin solution H.

H. Comparison solution containing pure oxyhaemoglobin.

G. Control. Same solution as F, non-irradiated.

F. Irradiated haemoglobin solution containing 0·5 molar potassium iodide.

E. Control. Same solution as D, non-irradiated.

D. Irradiated oxyhaemoglobin solution containing 0·5 molar potassium iodide.

C. Control. Same solution as B, non-irradiated.

B. Irradiated oxyhaemoglobin solution containing no added chemicals.

A. A spectrum photograph of the white light employed.

Figure 1. The Effect of Potassium Iodide on the Production of the Methaemoglobin Spectrum.

Identical behaviour was found with solutions of blood haemoglobin from the sheep and guinea-pig respectively. Evidence that this product of haemoglobin transformation was methaemoglobin was provided: (1) By noting the dissimilarity between the spectra of solutions of acid haematin and this product of photochemical oxidation when present in equivalent concentrations. (2) By reduction with ammonium sulphide followed by complete oxygenation when the oxyhaemoglobin spectrum became apparent.

Experiment 7. In order to determine whether the acceleration of oxidation was effected by iodides other than that of potassium, or whether it was specific for this particular salt, ammonium iodide was also tried. This salt in 0·5 molar concentration was also shown to effect a more rapid oxidation of blood pigment than occurred in solutions irradiated in its absence.

The more salient features of the foregoing experiments are apparent in the series of spectrum absorption photographs reproduced in fig. 1. Since the greatest divergencies between the absorption spectra of oxyhaemoglobin and methaemoglobin solutions are to be found in the red region, pigment solutions of such concentration that the other portions of the spectrum were completely obscured were found to illustrate variation in the proportion of these two pigments most satisfactorily. A constant exposure time of two seconds was employed in obtaineach of the photographs, and consequently a true representation of the relative absorptions of the various solutions is provided.

A decrease in width of the white area corresponds to a widening of the 6,300 Å.U. absorption band, due to an increase in concentration of methaemoglobin.

CONCLUSIONS.

1. The addition of potassium iodide to haemoglobin in solution accelerates its photochemical conversion to methaemoglobin, such acceleration being more marked with increase in concentration of this salt.

2. A more rapid conversion is obtained with reduced haemoglobin than with oxyhaemoglobin.

3. No variation in the extent of conversion occurs in solutions buffered to a pH of 7·4 when compared with unbuffered solutions.

4. When free iodine is added to a haemoglobin solution, either in the presence or absence of potassium iodide, the nature and speed of the reaction remain unaffected.

5. From the observation that ammonium iodide exerts an effect similar to that of potassium iodide, it appears that the iodine anion is probably the sensitizing radicle in this transformation.

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